THE UNIVERSITY OF ALBERTA

STUDIES ON METABOLISM AND TRANSLOCATION OF TRYPTOPHAN AND INDOLE ACETIC ACID IN SEEDLINGS OF SEVERAL SPECIES

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

NOVEMBER, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on Metabolism and Translocation of Tryptophan and Indole Acetic Acid in Seedlings of Several Plant Species" submitted by Ronald Leslie Whitehouse in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Tryptophan was found to be the major indole compound diffusing out of excised epicotyls of seedlings of Phaseolus vulgaris and Phaseolus multiflorus. It was also found to be the major indole compound extractable with methanol from these plant tissues. Cotyledons of 5-day old seedlings of P. multiflorus contained approximately 70 μg "free" tryptophan/g fresh weight. Other unidentified diffusible and extractable indole compounds were detected.

Indoleacetic acid-C^{14} (IAA-C^{14}) and tryptophan-C^{14} were translocated acropetally in shoots following injection of small amounts into cotyledons of P. multiflorus and endosperms of Zea mays seedlings. This translocation occurred in living tissue and not in the xylem. Extensive vascular systems in cotyledons of seedlings of P. multiflorus and P. vulgaris were shown.

A strict basipetal polarity of transport of IAA-C^{14} was shown in translocation experiments using Z. mays coleoptile and P. multiflorus epicotyl segments. Tryptophan-C^{14} was not actively translocated in either direction in such experiments. Passive diffusion from donor agar blocks into tissue for a limited distance, and diffusion in xylem vessels through the whole length of tissue segments was demonstrated with dye. Following administration of IAA-H^{3} from donor blocks, radioactivity was shown, by tissue autoradiography, to be present in all types of tissue in P. multiflorus epicotyls.

Microbial contamination was shown to be a problem in metabolic studies with tissue incubations of long duration. Microorganisms washed from surfaces of plant tissue were shown to metabolize tryptophan-C^{14}.

ACKNOWLEDGMENTS

The encouragement, enthusiasm and always available help of my supervisor, Professor Saul Zalik, are gratefully acknowledged. Many helpful discussions with my colleague, Dr. R. A. Fletcher, were appreciated. I would like to thank Mr. M. Stewart and Mr. J. Magus for their technical assistance, and Mrs. Gladys Kantor for help in preparation of sections for anatomical study. Thanks are due to Miss Leona Nordlie for her expert typing of the thesis. The years of patience and understanding given to me by my wife, Diane, are gratefully appreciated. Thanks are expressed to the University of Alberta and the National Research Council of Canada for providing financial assistance for this work.

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INTRODUCTION

Indoleacetic acid (IAA) is considered to be the major plant hormone. Although it has been investigated for several decades, the way in which it brings about its remarkable effects on plant growth and development are far from being fully understood.

Since etiolated seedling shoots must derive their organic nutrients from the storage material of the seed, either IAA itself or a compound from which IAA may be formed must be translocated to the shoot. But one of the intriguing properties of IAA in plants is that it is unable to be translocated acropetally in most young seedlings studied. Therefore it seems unlikely that IAA in the shoot is derived directly from the storage material of the seed, in fact, IAA has not been found among the endogenous compounds being translocated acropetally in seedling shoots. In view of this it seems likely that either a precursor or a complex of IAA not susceptible to the polar transport mechanism is translocated to the tip where IAA is produced from it.

The fact that the growing shoot tip is able to produce IAA from tryptophan suggests that this compound might be the translocated precursor. But the IAA content of storage tissue of seedlings generally increases upon germination. In view of this, the formation of a complex involving IAA as the translocatable form has been implicated. Thus it is not yet clear in what form the precursor is translocated acropetally.

The experiments reported in this thesis were designed with these facts in mind. For convenience the studies are grouped under four separate sections.

LITERATURE REVIEW

Auxin Transport

It has been known for a long time that many of the physiological and morphogenetic controls of plant growth and development are under the influence of the compound indoleacetic acid (IAA). For example IAA is produced in the apical growing regions of the shoot and is transported away from the tip where it can stimulate newly divided cells to elongate and thus cause elongation of the shoot itself. Another example of the morphogenetic control of plant growth and development is the phenomenon of apical dominance whereby IAA transported downwards from the shoot apex, prevents lateral bud growth. Distance from the apex is related to concentration of IAA since the concentration of IAA decreases with distance from the tip (e.g. Went and Thimann, 1937; Leopold and Lam, 1962; Scott and Briggs, 1962, 1963). Thus lateral buds may grow out only if the IAA concentration is below a certain level. In addition to the actual decrease in concentration from the tip downwards, the ability of the tissue to transport IAA decreases with distance from the tip (van der Weij, 1932; Jacobs, 1950, 1961; Scott and Briggs, 1962; Leopold and Lam, 1962; Leopold, 1963). Auxin itself maintains the tissue's ability to transport auxin, for if the apex is removed, the ability of the tissue to transport auxin is depressed (Leopold and Lam, 1962).

It is obvious from the two examples given above that different processes may be controlled or affected by different ranges in concentration of IAA. Another example is the fact that cell elongation

in roots is stimulated by a much lower concentration than the same process in shoots, in fact a concentration of IAA which is stimulatory to shoot elongation may be above the active range for and be inhibitory to root elongation (e.g. Thimann, 1937; Leopold, 1955, 1964).

From the foregoing and other evidence it has been concluded that IAA itself plays a major part in the control of plant growth and development.

One of the interesting properties associated with IAA is the polarity of its transport. It has been demonstrated that in many vegetative shoots, particularly those of monocots, IAA is translocated only in a basipetal direction (e.g. Went, 1928; van der Weij, 1932; Skoog, 1937; Jacobs, 1950; Leopold and Lam, 1961; Goldsmith and Thimann, 1962). Hertel and Leopold (1963) found active transport to occur in stems, leaves, coleoptiles, petioles and roots. They also found that active transport occurred in bryophytes and pteridophytes. In other cases, although IAA was translocated acropetally, the ability of the tissue to translocate it in this direction was less than that in a basipetal direction (e.g. Jacobs, 1954, 1961; McCready and Jacobs, 1963; Naqvi and Gordon, 1965). These workers obtained ratios of 3:1 for basipetal to acropetal transport. However, in flowering and fruiting stems the polarity is generally weakened and considerable amounts have been reported to be translocated acropetally (Leopold and Guernsey, 1953; Naqvi and Gordon, 1965; Sastry and Muir, 1965). For the polarity studies referred to above the tests were conducted on excised segments. On the other hand, Homan (1964), who administered IAA-C^{14} to intact pedicels of cucumber, found that parthenocarpy was induced and label moved into the developing fruit.

Using Convolvulus root segments Bonnett and Torrey (1965) found acropetal transport (towards the root tip) to be greater than basipetal transport (ca. 7:1).

Evidence for acropetal transport of endogenous auxin in the intact plant was obtained by Thimann and Skoog (1934) who found that the lateral bud immediately above a rapidly developing lateral bud in Vicia Faba was strongly inhibited. This inhibition was interpreted as being due to acropetal transport of auxin from the developing bud.

Under certain conditions IAA is able to be translocated acropetally, for example if the plant is treated with higher than physiological concentrations, or if IAA unnaturally gains access to the transpiration stream (Thimann, 1948; Leopold, 1961). Acropetal movement in woody stems has been reported by Oserkowsky (1942). Using sections of stem from apple and pear during the resting period he found acropetal transport of applied low concentrations of IAA. He suggested that the transport which operates both basipetally and acropetally is probably a diffusion process through the lumina of dead cells and through cell walls.

In translocation studies using tissue segments and IAA-C^{14} in agar blocks, the tissue takes up label irrespective of whether the labelled donor block is placed on the acropetal or the basipetal end. Some of the IAA may then be extracted by removing the labelled blocks and applying plain ones. Thimann (1964) states that this auxin probably simply diffuses into "free space" or perhaps cell walls, and

that the process is quite distinct from active transport. The uptake of IAA by the tissue occurs even in an atmosphere of nitrogen in which polar transport is greatly decreased. Recently, however, Naqvi et al. (1965) found considerable active transport of applied IAA-C^{14} in corn coleoptile segments even under anaerobic conditions. The amount transported anaerobically was about half that under aerobic conditions. Uptake of label by the tissues under anaerobic conditions also was about half that taken up aerobically. Consequently, the proportion of label absorbed which was translocated through to receiver blocks was the same in both cases.

An interesting demonstration of xylem transport of auxin was reported by Snow as long ago as 1929. Oserkowsky (1942) was also able to collect endogenous auxin from xylem vessels (i.e. the woody part of the stem with bark and cambium removed) from actively growing twigs. Endogenous auxin movement in the xylem in roots was also found by Pilet (1951).

Apparently several types of tissue are able to translocate IAA in a polar manner. Went and Thimann (1937) and Hertel and Leopold (1963) found such transport in parenchyma in oat and corn coleoptiles respectively. According to Went and Thimann (1937) and Leopold (1955, 1961) any metabolizing tissue including parenchyma, phloem, and cambium is capable of polar transport of auxin. Avery (1935) found that in Nicotiana leaves auxin moved primarily through the vascular bundles of the veins.

Biosynthesis of IAA

There appear to be several pathways for the biosynthesis of IAA in plants but the common precursor is tryptophan. IAA has been shown to occur in many higher plants and in addition it is also produced by many of the lower ones including bacteria (e.g. Stowe, 1955; Kaper and Veldstra, 1958; Kent and Gortner, 1960; Larsen et al., 1962; Magi et al., 1963; Klungsöyr, 1964) and fungi (see review by Gruen, 1959).

A wide variety of plants and tissues have been shown to be able to convert tryptophan to auxin, e.g. leaf, bud, stem, coleoptile, epicotyl, pollen, ovary, embryo, fruit, endosperm, cotyledon, root, crown gall and callus tissues and tissue cultures (Wildman et al., 1947; Tsui, 1948; Galston, 1949; Gordon and Nieva, 1949; Gustafsen, 1949; Stehsel and Wildman, 1950; Larsen, 1951; Gordon, 1954; Dannenburg and Liverman, 1957; Gordon and Paleg, 1957; Gordon, 1961; Gordon and Paleg, 1961; Ogasawara, 1961a, b; Wightman, 1962, 1964; Pilet, 1964). Gordon (1954, 1961) states that IAA production is associated with regions of the plant with high metabolic activity, for example, the rapidly growing shoots and roots, germinating endosperms and cotyledons. Wildman and Bonner (1948) found more activity for the conversion of tryptophan to IAA in the tip than in other parts of the Avena coleoptile. Gordon points out that the production of IAA is partly determined by the availability of tryptophan in a highly competitive system for tryptophan utilization.

Tryptophan has been shown to be present in fairly high concentrations in several organs and plants, e.g. in Brussels sprouts (Kutáček et al., 1959), in barley (Pleshkov, 1959) (see Fawcett, 1961). Nitsch (1955) found that the maximum level of auxin in strawberry achenes occurred at the same time that tryptophan was at its highest level in the receptacle; 12 days after pollination there was a rapid rise in auxin in the achenes and tryptophan in the receptacle and then a sharp drop in both. Of the seven growth promoting substances extracted from the achenes, one was found to be IAA. The maximum amount of tryptophan found was 17 mg/100 g dry weight. In potato tubers, Szalai (1959) found a rapid increase upon sprouting, the highest concentration of free tryptophan reported being 26.4 mg/100 g. A concentration of 0.25 mg/100 g was found in young plants, the leaf-stem and flowering tip of tomato plants by West (1959). Nitsch and Wetmore (1952) determined the free L-tryptophan content of various parts of seedlings of Lupinus albus and obtained the following distribution:

Plant part	mg/100 mg dry weight
apical meristem	140.0
leaf primordia	60.6
unfolded hairy leaves	38.6
large expanded leaves	16.9
epicotyl	69.6
cotyledons	276.6

It can be seen that the cotyledons and the apical meristem possess by far the highest concentrations of free tryptophan on a dry weight basis.

Lawrence and Grant (1963) found 17.6 mg/100 g dry weight of tryptophan in 5-day old pea seedling cotyledons, but only a trace in the shoot tips. In the shoot shafts, however, there was approximately 90 mg/100 g.

Source of IAA in the Shoot Apex

Went and Thimann (1937) found that it was impossible to obtain even the slightest amount of auxin from the apical cut surfaces of coleoptile or mesocotyl stumps when connected with the seed even in the earliest stages of germination. Gordon (1954, 1961) also states that it is clear that the auxin as such which increases in the seed upon germination does not move to the apex for redistribution as a correlative agent.

Skoog (1937) also was unable to detect any auxin transported from the seed acropetally. But he was able to collect in agar blocks applied to cut coleoptiles a substance which gave a curvature in the Avena test only a long time after application (10 - 20 hrs.). This suggested the transport upwards of a precursor which was able to be converted to auxin by Avena coleoptiles only a long time after application. Removal of the remains of the endosperm also was found to curtail auxin production by the tip and regeneration of the physiological tip. The response of the Avena curvature test to this substance was found to be similar to the response to tryptophan.

Thimann and Went (1934) also reported that tryptophan induced a slow curvature in the Avena test.

Went and Thimann (1937) and Skoog (1937) suggested that tryptophan might be the precursor of IAA which is translocated to the tip. The fact that the tip is able to use tryptophan for conversion to IAA (see e.g. Wildman and Bonner, 1948) is supporting evidence for this.

Thimann and Went (1934) found that tryptophan stimulated growth when it was applied to the base, but not when it was applied to the apex of Avena coleoptiles. Stewart (1941) applied 2% tryptophan in lanolin paste and agar blocks unilaterally to coleoptiles of Avena, 15 mm below the tips, and found that definite curvature occurred. However, in view of the fact that he questioned the purity of the tryptophan samples used and because of the high concentration employed, the evidence must be regarded as equivocal. A more recent study by Schrank and Murrie (1962) showed that tryptophan-C^{14} was not actively translocated in Avena coleoptile sections, either acropetally or basipetally, although it was taken up by the tissue slowly and for a limited distance from the source. Concentrations as high as 10^{-3}M applied to the basal ends of 17.5 mm coleoptile tips did not increase elongation over controls during a 20 hr growth period; in fact no radioactivity was extracted from the apical 5 mm.

Several German workers cited by Larsen (1951) and Gordon (1954, 1961) have reported on an auxin complex formed in the scutellum of corn. This complex was reconverted into auxin by Avena coleoptile tips (Tegethoff, 1951, as cited by Gordon, 1954).

Acropetal movement of an inactive precursor was also found by Voss. Voss (1938) suggested that auxin, formed in the endosperm, is bound in the scutellum as a complex, transported to the tip and reconverted there to auxin for redistribution downwards. Larsen (1951) and Gordon (1961) discussed the complex investigated by these workers. The complex, which could be obtained from tissues by diffusion or extraction and from which auxin was released enzymatically, had an apparent molecular weight higher than that of IAA. It was stable in H_2O_2 and hot alkali but was inactivated by acid. Diverse tissues such as roots, scutella, first internodes, tubers and green leaves were able to carry on this binding of auxin. It possessed no specificity of transport polarity and auxin was readily liberated from it by tissues active in auxin production.

During development of both monocot and dicot seeds, the content of free auxin generally increases and then decreases (e.g. Avery et al., 1942; Hatcher, 1945; Luckwill, 1953; Nitsch, 1950, 1952, 1955). Stehsel and Wildman (1950) attributed the drop in free auxin to the formation of the bound form. That auxin is present in a bound form in the storage material of the seed has been shown (e.g. by Avery et al., 1941; Berger and Avery, 1944; Hatcher, 1945; Stehsel and Wildman, 1950). Larsen (1951) considers that the auxin complex investigated by Avery and co-workers could be identical with the one investigated by the German workers.

Dark-grown seedlings rely for their organic nutrients entirely upon the storage material of the endosperm or cotyledons of the seed. Therefore, IAA present in the shoot must be derived from the seed. In view of the basipetal polarity of most young seedling

shoots it is unlikely that IAA itself moves acropetally into the shoots, unless of course it could be transferred acropetally in a way such that it was immune from the normal polar transport mechanism. This might be feasible through some sort of complex or derivative of IAA in which form it is translocated. Some complexes have already been referred to.

Various other complexes of IAA which should be mentioned are glucobrassicin and neoglucobrassicin present mainly in young cabbage leaves. Glucobrassicin, upon hydrolysis, yields 3-indoleacetonitrile (a precursor of IAA in some plants), glucose, sulphate, hydrogen sulphide and sulphur. Gmelin (1964) found, however, that these are completely lacking in cabbage seed.

Earlier Andreae and Good (1955, 1957) and Andreae et al. (1961) and Zenk (1961) found another IAA complex, indoleacetylaspartic acid, which they obtained from pea tissue upon the addition of IAA. They suggested that formation of this compound could be a detoxification mechanism and/or could regulate the supply of free IAA. That it occurs naturally in the tomato seedling has been shown by Row et al. (1961). Information on complexing of IAA with other small molecules such as glucose to form IAA-glucose has been provided by Zenk (1961, 1964). There is good evidence that this mechanism also is possibly a detoxification mechanism. Malonyl-D-tryptophan was also found to be a detoxification mechanism for D-tryptophan. Zenk suggests that to prevent further IAA production in a maturing fruit, some IAA precursor is transformed to D-tryptophan which is immobilized and detoxified as malonyl-D-tryptophan. This compound apparently accumulates in the

mature fruit. This prevents further production of IAA and the action of already formed IAA is prevented by production of the complexes indoleacetylaspartic acid and indoleacetyl glucose.

Another complex, involving IAA with ascorbic acid, ascorbigen, was detected in Brussels sprouts seeds two days after sowing, but could not be detected in dormant seeds (Kutáček et al., 1959). A similar increase in this compound was also found during the germination of legume seeds (Banerjee et al., 1958, as cited by Fawcett, 1961).

Whether these compounds are directly involved in the transport of auxin from the storage tissue of the seed to the actively growing, auxin producing regions of the shoot has so far not been shown.

GENERAL MATERIALS AND METHODS

Plant Material

The main plants used were Phaseolus vulgaris (var. Dutch brown beans), Phaseolus multiflorus (var. Scarlet Runner beans) and Zea mays (var. Morden).

Seedlings of Dutch brown beans and Runner beans were grown in previously moistened California mix in either 15 cm pots or flats and grown in the dark at 25 C. They were used between 5 and 7 days after planting. The only light which the plants received was a few seconds exposure while being transferred from one room to another.

In 7-day old seedlings of Dutch brown beans, the hypocotyl is long and spindly but the epicotyl is short and unfolded. To enhance uncurling and lengthening of the epicotyl, 5 to 7-day old seedlings were exposed to daylight for 1 day.

Corn seeds were treated with a 10% solution of commercial sodium hypochlorite (Perfex) for 10 min at room temperature in the dark and then rinsed for 3 hrs in running tap water (Gillespie and Thimann, 1963). They were then sown in previously moistened California mix in Pyrex dishes (28 x 36 cm), covered with a layer of about 0.5 cm of moist California mix and the dish covered with another inverted dish of the same size. On the second day after planting, when the seedlings were just beginning to emerge, they were given a red light treatment (15-watt bulb with a sheet of red plexiglass at a distance

of about 30 cm) for 3 hrs to enhance elongation of the coleoptiles (Briggs, 1963). Seedlings 3.5 - 4 cm tall were used on the third day after sowing.

Green Safelight

Manipulations to seedlings were carried out using a safelight constructed from a 15-watt Sylvania green fluorescent tube (peak transmission at 525 mμ, with the wavelength range at 50% maximum transmission being 508 - 550 mμ). Light transmitted from this was further screened by three layers of green Plexiglass No. 2092 and one of amber Lucite. Transmission of the filter was checked using a Beckman DK-1 spectrophotometer and was found to be zero at 500 and 560 mμ with a peak at 525 mμ. This range is similar to the range used by other workers, e.g. Withrow and Price (1957).

Preparation of Methanol Extracts of Plant Tissue

First extracts

Plant material was sliced into methanol (Nitsch, 1956) and ground using either a pestle and mortar or an Omnimixer. The preparations were then left for 2½ - 4 hrs at 3 C and were filtered using a Buchner flask and Whatman No. 1 filter paper. (In some cases several filtrations or the use of a finer filter paper was necessary to produce a clear filtrate.)

Second extracts

The residue obtained after extracting for a short period of time was further extracted with methanol either (a) for several days at 3° C, or (b) overnight with a Goldfisch extraction apparatus.

Third extracts

Residues were hydrolyzed in buffer, pH 9.5 (50 ml 0.025 M borax, 9 ml 0.1 M NaOH) at approximately 110° C for 20 min using an autoclave. The tissue and solution were then dried by evaporating off the solvent over direct heat. The residue was then extracted with methanol for several hrs at room temperature.

Chromatography

The extracts were evaporated down to small volumes (0.5 - 1 ml) under reduced pressure in a rotary evaporator at 40 - 45° C. The concentrated extracts were applied as bands on strips of Whatman No. 1 chromatography paper and dried by a stream of cold air. Difficulty was experienced in applying unpurified methanol extracts to the chromatograms because of the viscosity, stickiness and tendency for substances to precipitate out. Also if too much extract was applied to the paper strip in too narrow a band, the application could not be dried, but remained sticky and streaked on chromatographing. The chromatograms were developed using the descending technique in isopropanol:ammonium hydroxide (28%):water, 8:1:1 (Stowe and Thimann, 1954). Other solvents used will be specified.

Detection of Compounds on Chromatograms

Indole compounds were detected by spraying the chromatograms with a solution of p-dimethylaminocinnamaldehyde (DMAC) (Harley-Mason and Archer, 1958). This was prepared by dissolving 1 g DMAC in a mixture of 50 ml 95% ethanol and 50 ml 6N NCl. In some cases a strip

was taken from the edge of a chromatogram and sprayed and the rest of the chromatogram at the region of the indole compounds investigated was excised, and eluted with 3.5 ml methanol by shaking. The absorbance of this extract in the ultra violet (u.v.) region of the spectrum (240 - 300 mμ) was examined using a Beckman DK-1 spectrophotometer.

Radioactive regions of chromatograms were detected by a number of methods.

1. Chromatograms were scanned using a Nuclear-Chicago Actigraph II chromatogram scanner.

2. Radioautograms were made on Ansco Non-Screen X-ray film. Exposure time varied from 1 to 6 weeks. Films were developed in Kodak D-19 developer for 4 - 5 mins and were fixed for 10 min or longer in Kodak Fixer. They were then washed in running water for approximately 1 hour.

3. Chromatograms of low activity and also those containing tritium-labelled compounds were cut into 20 segments and the activity of these portions determined with a Nuclear-Chicago Liquid Scintillation Counter.

Tests on Synthetic Compounds Used

The authenticity of the synthetic IAA used was checked by comparison with a fresh sample purchased from Fisher Scientific Co. Absorption spectra in the infra-red and ultra-violet regions, R_f values in isopropanol:ammonium hydroxide (28%):water (8:1:1) and color reactions with DMAC were identical. R_f values of a number of indole compounds

chromatographed in several solvents are given in table I together with their colour reactions with DMAC.

Table I. R_f values and colour reactions with DMAC of synthetic indole compounds chromatographed in various solvents.

Indole compound	R_f value in solvent						Colour with DMAC
	A	B	C	D	E	F	
IAA	.82	.39	.73	.85 (.95)	.76	.83	purple
DL-Tryptophan	.40	.30	.14	.26 (.36)	.57	.66	purple
Indole acetonitrile	.87	.85	.86	.91	.82	.45	reddy purple
Indole carboxylic acid	.79	.33	.80		.80	.80	green
Tryptophol	.83	.85	.85	.89	.80	.58	reddy purple
Indole lactic acid	.73	.42	.35	.44	.68	.81	reddy purple
IAA-ethyl ester	.87	.89	.90	.90	.86	.52	purple
Indoleacetamide	.75	.74 (.43)	.73 (.58,.90)	.76	.73	.57	purple
Indole pyruvic acid	.70 streak	.35 (.42,.64)	.30 (.62)	.39 (.72,0)	.61 (.71)	.74 (.81)	purple
Indole butyric acid	.84	.55 (.79)	.84	.87 (0)	.81	.83	purple
Indole propionic acid	.82	.46	.79 (.90,0)	.85 (0)	.75	.83	purple
Tryptamine	.68	.75	.30	.48	.69	.11	purple
Indolealdehyde	.84	.85	.84				green
Indoleacetaldehyde	.58	.82	.54 (.92,0)		.67,.79	.51	pinky? green

A isopropanol:acetic acid:water (4:1:1)
B isopropanol:ammonium hydroxide (28%):water (8:1:1)
C isobutanol:methanol:water (80:5:15)
D n-butanol:ethanol (95%):water (4:1:1)
E 70% ethanol
F water

Rf values of minor secondary regions are given in parenthesis.

Radioactively-labelled Compounds

(Indole-3)-acetic acid-1'-C^{14} was purchased from California Corporation for Biochemical Research and had a Specific Activity of 13.3 mc/mM.

DL-Tryptophan-1-C^{14} (alanine-3-C^{14}) purchased from the same company had a Specific Activity of 32.5 mc/mM.

Indole Acetic Acid-T (Generally Labelled) purchased from Merck, Sharp and Dohme of Canada Ltd., had a Specific Activity of 112 mc/mM.

Determination of Radioactivity in Extracts

Extracts were applied to aluminum planchets and dried by heating with an infra red lamp. Radioactivity was then determined using a Nuclear-Chicago Model D-47 Gas Flow Counter, correction being made for background.

SECTION A. ENDOGENOUS INDOLE COMPOUNDS

This section deals with diffusible and extractable endogenous indole compounds in Phaseolus sp. Since this work originally stemmed from the work of Fletcher and Zalik (1963, 1964, 1965), the plant first investigated was Phaseolus vulgaris (var. Dutch brown beans) as this was the plant material used in their studies.

Diffusible Indole Compounds in Phaseolus vulgaris

Dark-grown 6-day old seedlings were kept in the light for 1 day to enhance lengthening and unfolding of the epicotyls. Epicotyls were excised and filter paper discs approximately 6 mm diameter were placed on the cut epicotyls. Treated plants were then kept for five hours in a humid atmosphere to prevent the discs' drying out. The discs were then removed, dried and placed into slits cut in strips of Whatman No. 1 chromatography paper, thus:

In order to increase the concentration of extract applied to the chromatograms, several discs were combined on each strip. The chromatograms were developed overnight.

The results given in table II were obtained.

Table II. R_f values and DMAC reactions of compounds collected in paper discs from cut epicotyls of Phaseolus vulgaris and chromatographed in isopropanol:ammonium hydroxide: water (8:1:1).

Colour reaction with DMAC	Mean R_f*
pink	0.08
blue	0.22
purple	0.34
pale grey?	0.70
pale pink?	0.88

* mean of 3 chromatograms each containing 5 discs.

Diffusible Indole Compounds in Phaseolus multiflorus

The main concern of this study was with endogenous indole compounds in the shoot which are derived from the cotyledons. However, Phaseolus vulgaris (var. Dutch brown beans) was not a suitable plant for these studies because of its epigeal character and the fact that with etiolated plants the epicotyl does not elongate until the hypocotyl is long and spindly and the plants very weak. Also because it was required that the shoot be dependent upon food reserves in the cotyledons, the light treatment necessary to enhance epicotyl unfolding and elongation was undesirable. Therefore another species of the same genus, Phaseolus multiflorus Willd. (Scarlet Runner beans) which is hypogeal in its germination, was investigated.

Epicotyls of 5-day old dark-grown Runner bean seedlings were excised and blocks of 1.5% agar placed on the cut surfaces to collect the diffusate both up from the cotyledons and down from the tip. These were left for 2 - 4 hrs in a humid atmosphere in the dark. The agar blocks were then removed, pooled in treatments and extracted with 95% ethanol overnight at 3° C (Kuraishi and Muir, 1964). The extracts were decanted from the agar blocks and fresh solvent added for an additional extraction period of 3 hrs. Extracts were combined and chromatographed on duplicate 4 cm strips of Whatman No. 1 chromatography paper. The procedure was repeated using 6-day old plants, and filter paper discs instead of agar blocks. The filter paper discs containing diffusate were not chromatographed directly because it was desired to concentrate the extracts, so these were extracted as were the agar blocks. Chromatograms were sprayed with DMAC and results given in table III were obtained.

Table III. R_f values and DMAC reactions of diffusible compounds, which were collected from cut epicotyls of Phaseolus multiflorus and chromatographed in isopropanol:ammonium hydroxide:water (8:1:1)

R_f values of compounds detected in					
agar blocks			paper discs		
Colour with DMAC	Diffusing		Colour with DMAC	Diffusing	
	Down	Up		Down	Up
Pink	0.06	0.06	Pink	0.0	0.0
			Pink	0.09	0.09
Purple	0.10	0.14	Pale blue-purple	0.13	0.12
Purple	0.30	0.31	Pale blue-purple	0.31	0.31
Pale grey?	0.64	0.65	V. pale grey?	0.64	0.64
Pale pink	0.93	0.93	Pale pink	0.92	0.93

The same compounds were collected from Runner beans as from Dutch brown beans. Using paper discs two pink bands at low R_f values were obtained, one at the origin and the other at R_f 0.09 whereas with agar blocks only one pink band at R_f 0.06 was obtained. The compounds collected diffusing up from the base and down from the tip were the same. Although both agar blocks and paper discs were satisfactory for collecting diffusate, chromatograms from the same number of plants treated with agar blocks revealed more intense bands than those treated with paper discs.

Indole Compounds Extracted from *Phaseolus multiflorus*

It was decided to investigate whether IAA could be detected in extracts of *P. multiflorus* using the method of Fletcher and Zalik (1963, 1964) for *P. vulgaris* and also to investigate what other naturally occurring indole compounds could be detected with this method.

Six-day old dark-grown seedlings were washed to remove soil and the seed coats removed. Plants were divided into shoots, cotyledons and hypocotyls plus roots. The weighed plant parts were extracted with methanol for 2 hrs and the extracts chromatographed on 10 cm strips of chromatography paper. After developing the chromatograms a quarter-inch strip was cut from the edge of each chromatogram and sprayed with DMAC to locate indole compounds. An intense purple band was located at a mean R_f of 0.29 (see table IV) and a less intense blue-purple band at a mean R_f of 0.12, in addition to the pink bands running not far from the origin.

Table IV. R_f values of compounds which react purple with DMAC, extracted from seedlings of Phaseolus multiflorus with methanol and chromatographed in isopropanol:ammonium hydroxide:water (8:1:1) (all values are means of duplicate estimations).

Tissue	R_f values of compounds reacting purple with DMAC		µg "IAA" based on absorption of B at 280 mµ	
	A Blue-purple	B Intense purple	/plant part	/g fresh weight
shoot	0.12	0.29	170	52.4
cotyledon	0.11	0.30	214	56.0
roots + hypocotyls	0.12	0.29	224	76.0
	µg "IAA"/plant		812	

The rest of each chromatogram at the location of the intense purple area was cut into small pieces and eluted with methanol. The absorption of the eluate was measured in the u.v. range 240 - 300 mµ. A typical curve produced is illustrated in figure 1 together with curves for synthetic IAA and DL-tryptophan. The curve of the unknown plant compound is typical of compounds containing an indole nucleus.

It was at first thought that this compound was IAA because of its R_f value near that of IAA in the chromatography solvent used, its colour reaction with DMAC and its u.v. absorption spectrum. Using the method of Fletcher and Zalik (1963) and their standard curve, the IAA concentrations were calculated using the absorbance at 280 mµ. These values are also given in table IV.

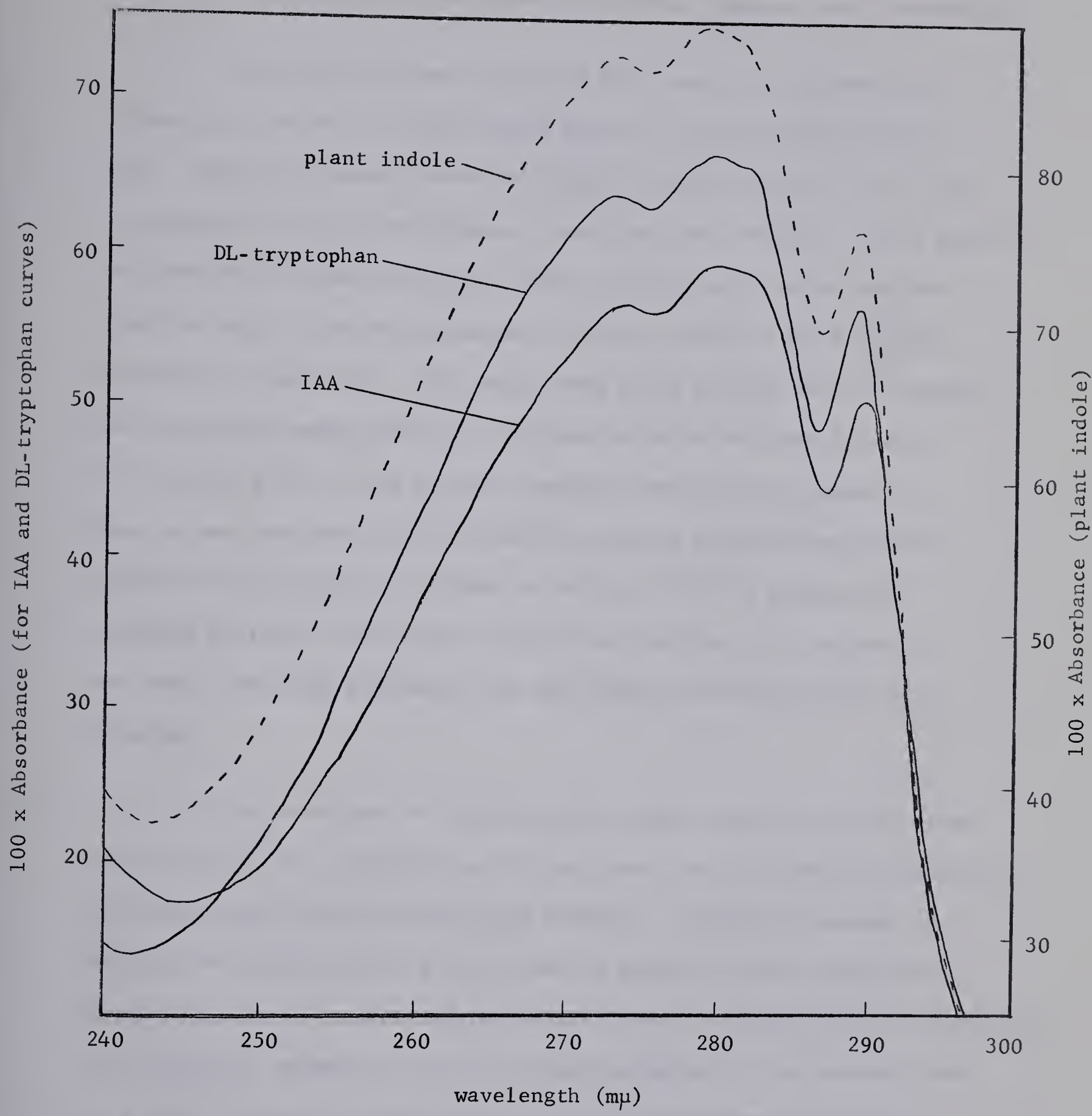


FIG. 1. Absorption curves for IAA, DL-tryptophan and compound extracted from Runner bean shoots with methanol (R_f 0.29 in isopropanol:ammonium hydroxide (28%):water, 8:1:1).

The concentrations obtained were unexpectedly high. Therefore, further investigations on the identity of this compound were undertaken.

Other extracts were prepared from shoots, cotyledons and hypocotyls plus roots of dark grown plants of various physiological ages. Duplicate chromatograms were made from each extract and on one set synthetic IAA was superimposed over the plant extract. After spraying the developed chromatograms with DMAC an additional purple band was observed only on the chromatograms to which synthetic IAA had been applied (see table V). This purple band had a mean R_f of 0.39 whereas the two purple bands found in the plant extracts had mean R_f values of 0.10 and 0.28 in this solvent (refer to bottom row of table V). Thus it was concluded that the indole compound producing an intense purple colour reaction with DMAC at an R_f of 0.28 in isopropanol: ammonium hydroxide (28%):water (8:1:1) was not IAA. No evidence of any purple reacting compound at an R_f region of the IAA (0.39) was observed.

The procedure was repeated many times using both Dutch brown beans (though of a different batch from those used by Fletcher and Zalik) and Runner beans with the following changes: varying the amount of material extracted; varying the volume of methanol used; varying the extraction times; evaporating the extract down to dryness and re-dissolving the residue in methanol instead of simply evaporating the extract down to a small volume for application to the chromatograms; using 7% ammonium hydroxide instead of 28% in the developing solvent. One other variation was the use of 25 6-day old apices of Runner beans including young leaves for extraction.

Table V. Compounds reacting purple with DMAC extracted with methanol from Phaseolus multiflorus seedlings of various physiological ages and chromatographed in isopropanol:ammonium hydroxide (28%):water (8:1:1)

Tissue	Fr. wt. (g)	Equivalent amount plant material (g) chromato-graphed	R_f values of purple bands with DMAC in: Plant extract alone		Plant extract + IAA		
I. Plants with epicotyls 7.5 - 11.5 cm long							
a. epicotyls	4.4	2.2	0.11	0.30	0.10	0.28	?
b. cotyledons	10.0	2.5	0.12	0.30	0.08	0.23	0.30
c. hypocots. and roots	3.0	2.0	0.10	0.28	0.09	0.26	0.41
II. Plants with epicotyls 3 - 6.5 cm long							
a. epicotyls	2.9	1.9	0.12	0.28	0.10	0.26	0.38
b. cotyledons	10.2	2.6	0.11	0.28	0.10	0.24	0.34
c. hypocots and roots	3.0	2.0	0.09	0.27	0.09	0.28	0.44
III. Plants with epicotyls 2 cm long							
a. epicotyls	1.45	1.45	0.10	0.29	0.10	0.28	0.41
b. cotyledons	10.2	2.6	0.09	0.27	0.08	0.26	0.34
c. hypocots. and roots	2.8	1.8	0.10	0.29	0.09	0.28	0.42
IV. Plants with epicotyls <1.5 cm long							
a. epicotyls	1.1	1.1	0.10	0.27	0.09	0.26	0.38
b. cotyledons	10.2	2.56	(0.20)		(0.16)		
c. hypocots. and roots	2.4	2.4	0.10	0.28	0.10	0.30	0.45
Mean R_f			0.10	0.28	0.09	0.27	0.39

The regions on the chromatograms where IAA would be expected to chromatograph as obtained from the R_f values of synthetic IAA run with the samples were eluted with methanol and the u.v. absorption spectra examined. No evidence of IAA or an indole compound was obtained in any of these extracts. In all cases where IAA was superimposed on the plant extract, a band not appearing on chromatograms of plant extract alone was detected upon spraying with DMAC.

Investigation of the other indole compounds in the plant extracts, particularly the main compound reacting purple with DMAC was carried out. It was suspected that this compound was tryptophan.

Runner bean shoots and cotyledons were extracted separately with methanol for 3 hrs at 3^{o} C (first extract) and the residues after filtering were extracted with methanol in a Goldfisch extraction apparatus overnight (second extract). Eight chromatograms were prepared from each extract on 10 cm strips of Whatman No. 1 chromatography paper, duplicates to be run in each of four chromatography solvents. To one of each duplicate, IAA and DL-tryptophan were superimposed over the plant extract on the origin, a line 2.5 cm long of tryptophan being applied on one end of the origin and a line 2.5 cm long of IAA on the other end. In addition for each solvent run a 4 cm strip of IAA and tryptophan was run. The four solvents used were:

A. isopropanol:ammonium hydroxide (28%):water (8:1:1).

B. isopropanol:acetic acid:water (4:1:1).

C. isobutanol:methanol:water (80:5:15).

D. butanol:ethanol (95%):water (4:1:1).

After development the chromatograms containing synthetic IAA and tryptophan (i.e. IAA and tryptophan alone and plant extract plus IAA and tryptophan) were sprayed with DMAC. In addition a strip about .5 cm was taken off the edge of the chromatograms of the plant extracts alone and this also was sprayed. The results obtained are shown in figures 2 - 5.

Tryptophan co-chromatographed with the compound reacting deep purple with DMAC in all 4 solvents. The colour reaction of tryptophan with DMAC is also purple. The rest of each unsprayed chromatogram was eluted with methanol in the regions corresponding to the coloured bands in the detection strips. The absorption spectra in the u.v. region from 240 mμ to 300 mμ were obtained for each of these extracts. Typical indole curves were obtained for the compound reacting deep purple with DMAC. For the other bands, suggestions of the indole curve were obtained in some cases though generally they differed in several respects. This is not surprising in view of the fact that many compounds present in the crude extracts will have co-chromatographed with these other compounds which generally travelled a shorter distance than the main compound. Chromatograms developed in isopropanol:ammonium hydroxide:water (8:1:1) and sprayed with ninhydrin and a spray reagent for sugars (0.615 g p-anisidine and 0.83 g phthalic acid in 50 ml ethanol) showed that these two types of compounds had chromatographed over the area under investigation.

After use in the spectrophotometer the methanol solutions were allowed to concentrate in the air at room temperature and were then re-chromatographed using different solvents from those in which

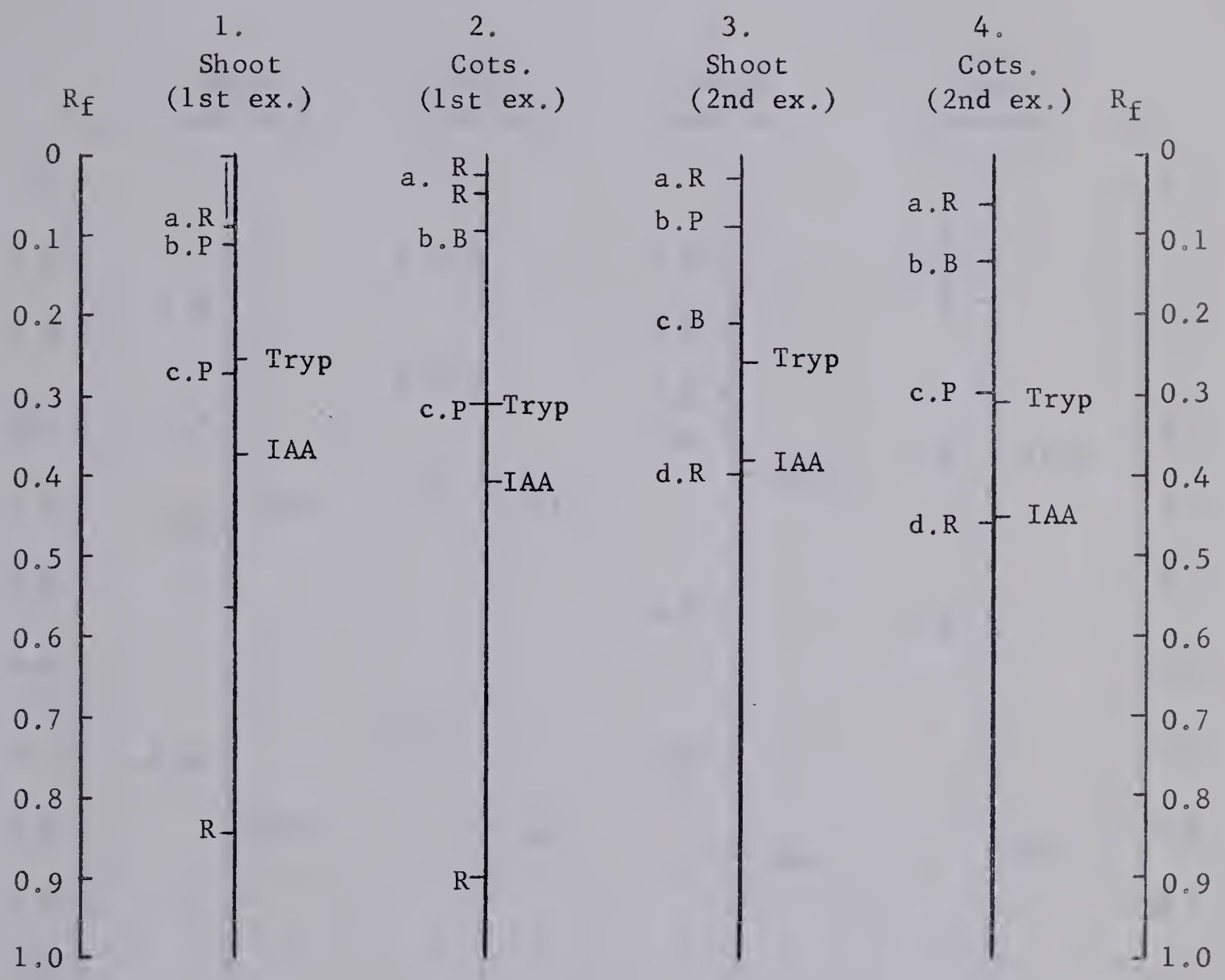


FIG. 2. Mean R_f values and DMAC reactions of compounds extracted with methanol from seedlings of Phaseolus multiflorus and chromatographed in isopropanol:ammonium hydroxide:water (8:1:1).
(Synthetic tryptophan (Tryp) and IAA. Colour with DMAC, R = red or pink, P = purple, B = blue. Small Latin letters refer to regions extracted for re-chromatography and relate to table VI.)

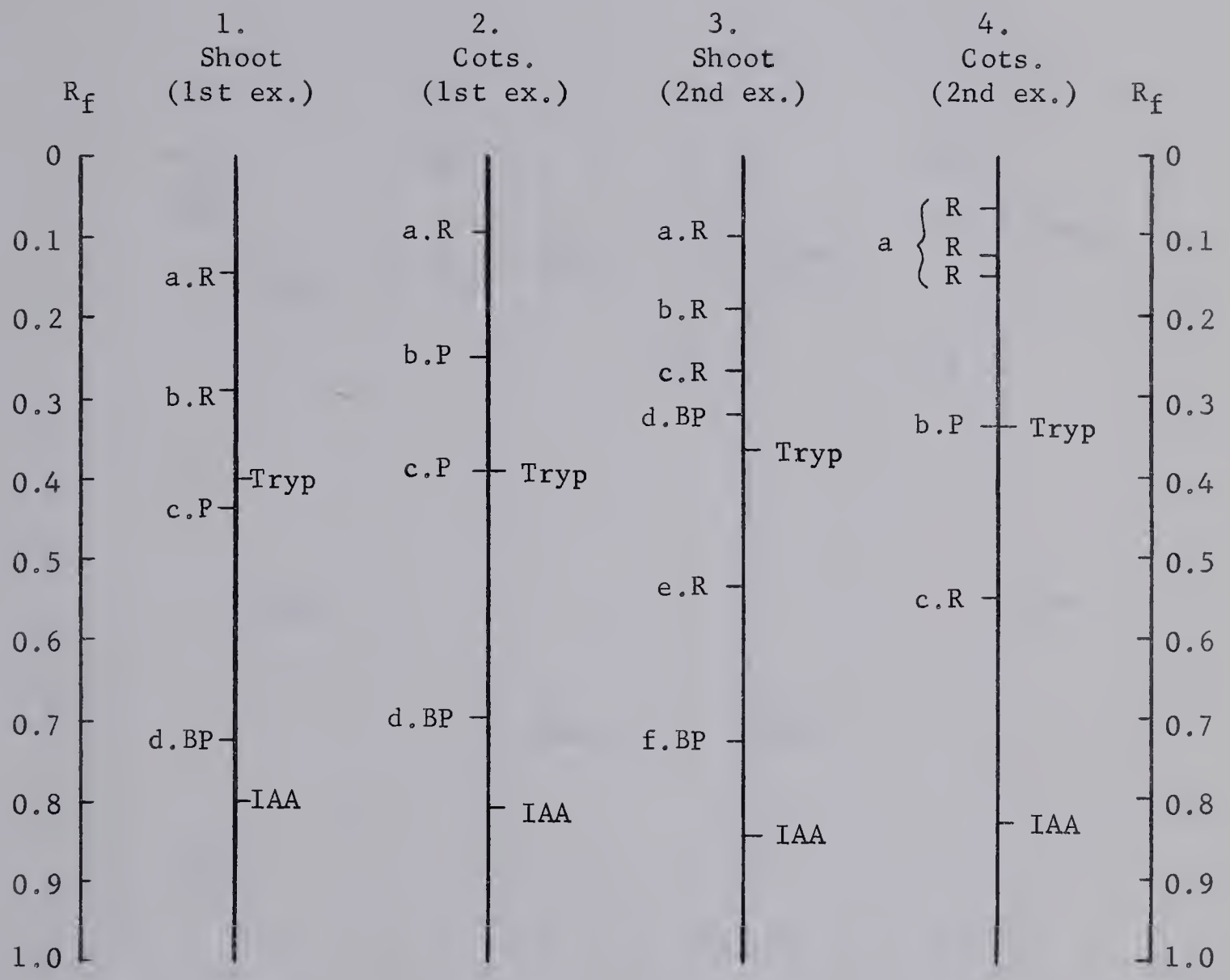


FIG. 3. Mean R_f values and DMAC reactions of compounds extracted with methanol from seedlings of Phaseolus multiflorus and chromatographed in isopropanol:acetic acid:water (4:1:1).
(Synthetic tryptophan (Tryp) and IAA. Colour with DMAC, R = red or pink, P = purple, B = blue. Small Latin letters refer to regions extracted for re-chromatography and relate to table VII.)

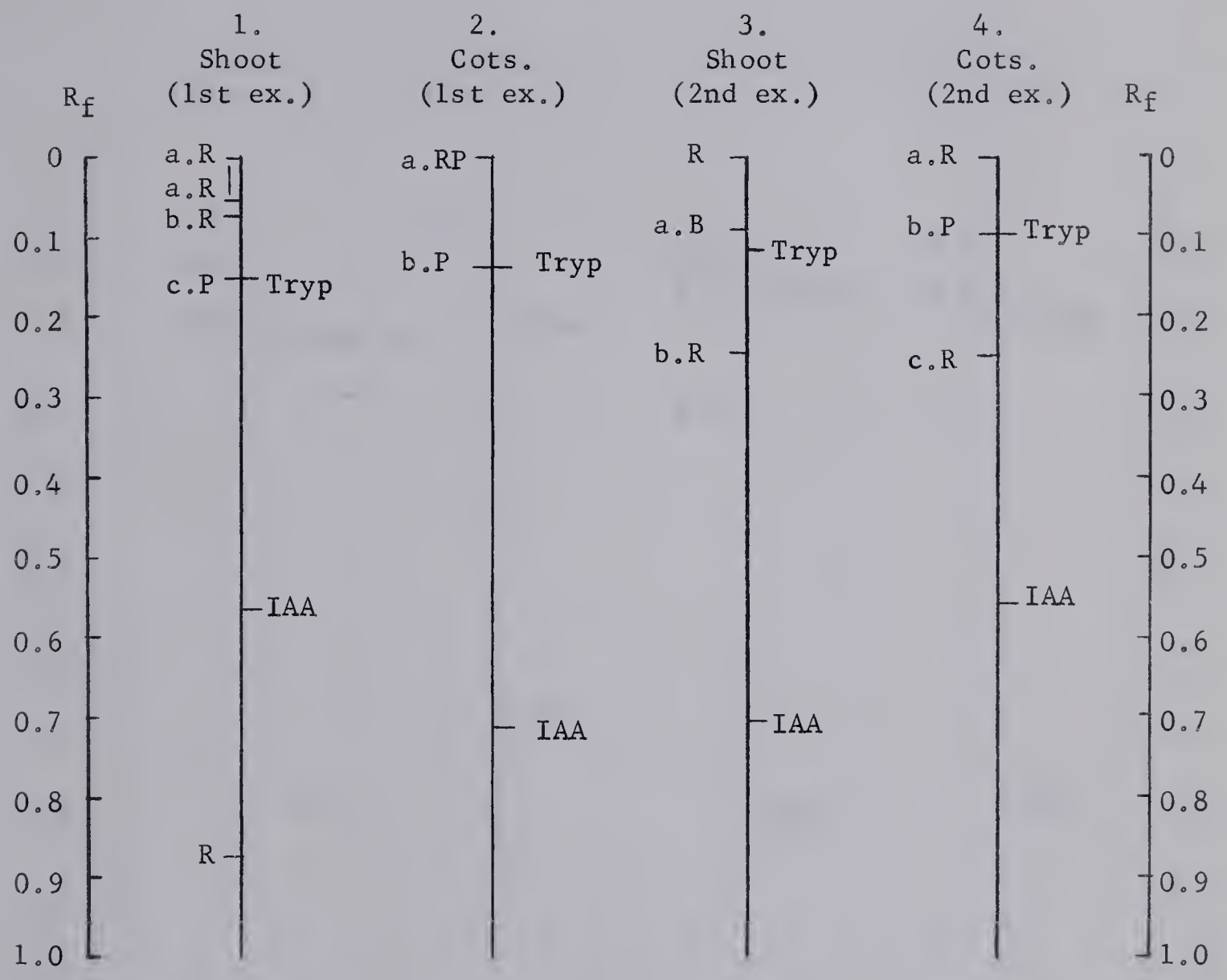


FIG. 4. Mean R_f values and DMAC reactions of compounds extracted with methanol from seedlings of Phaseolus multiflorus and chromatographed in isobutanol:methanol:water (80:5:15).
(Synthetic tryptophan (Tryp) and IAA. Colour with DMAC, R = red or pink, P = purple, B = blue. Small Latin letters refer to regions extracted for re-chromatography and relate to table VIII.)

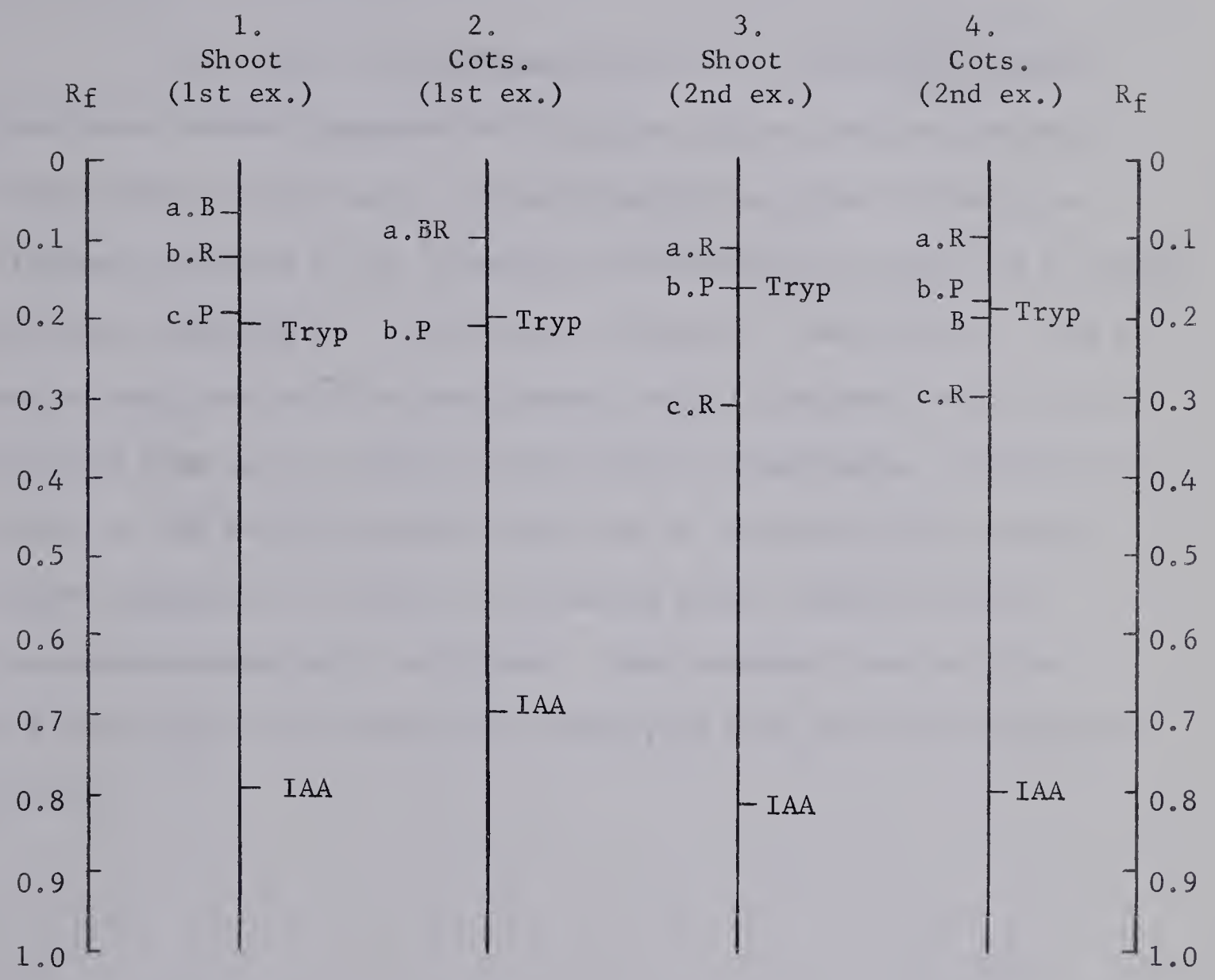


FIG. 5. Mean R_f values and DMAC reactions of compounds extracted with methanol from seedlings of Phaseolus multiflorus and chromatographed in n-butanol: ethanol:water (4:1:1).
(Synthetic tryptophan (Tryp) and IAA. Colour with DMAC, R = red or pink, P = purple, B = blue. Small Latin letters refer to regions extracted for re-chromatography and relate to table IX.)

they were run initially, though using 3 of the 4 solvents used for the initial separation. This time each eluate was applied as a single spot. After development the chromatograms were sprayed with DMAC and the results obtained are given in tables VI - IX.

This method of re-chromatography was carried out because there were several compounds with similar colour reactions on each chromatogram. With simply chromatographing the crude extracts in different solvents it was therefore not possible to relate the R_f values of these compounds in the different solvents. From tables VI - IX it can be seen that on re-chromatography several compounds were usually detected from one R_f region of the initial chromatogram. Only the R_f values of the major compounds were used in calculating the mean R_f values summarized in table X. The major purple compound again co-chromatographed with tryptophan. The compounds reacting pink and blue-purple with DMAC do not correspond with any indole compound tested.

Table VI. Colour reactions with DMAC and mean R_f values in isopropanol: acetic acid:water (4:1:1) of regions of chromatograms of Phaseolus multiflorus extracts initially developed in isopropanol:ammonium hydroxide:water (8:1:1)

Extract identification and data from fig 2			Colour reaction with DMAC and R_f value in isopropanol:acetic acid:water (4:1:1)				
Extract	Colour with DMAC	R_f region in iP:NH_3:H_2O					
1a	red pink	0-0.09	R.06	R.20		R.47	
1b	purple	0.12	R.06	R.21			P.70
1c	dark purple	0.28			P.28		
2a	pink	0.03	R.06	R.20			
2b	bluey	0.10	R.05	R.18	P.27	R.44	B.64
2c	dark purple	0.31			P.25		
3a	pink	0.03	R.09	P.19		R.46	
3b	blue pink	0.09	R.05	R.19		R.46	
3c	bluey	0.21			P.17	R.45	
3d	pink	0.40				R.45	
4a	pink	0.06	R.07	R.18		R.46	
4b	blue	0.13	R.05	R.18			
4c	purple	0.30			P.20	R.45	
4d	pink	0.46				R.45	
Mean R_f of purple compound		0.28			0.20		
Tryptophan		0.28			0.21		
IAA		0.40			0.82		

R = red or pink

P = purple

B = blue

Table VII. Colour reactions with DMAC and mean R_f values in butanol:ethanol:water (4:1:1) of regions of chromatograms of Phaseolus multiflorus extracts initially developed in isopropanol:acetic acid:water (4:1:1)

Extraction identification and data from fig 3			Colour reaction with DMAC and R_f value in butanol:ethanol:water (4:1:1)				
Extract	Colour with DMAC	R_f region in iP:acetic:H_2O					
1a	pink	0.14	R.02	R.13		R.32	
1b	pink	0.29	R.03		P.17		
1c	dark purple	0.43		B.15	P.20		
1d	blue purple	0.72	B.04				
2a	pink	0.09	R.01			R.30	
2b	blue purple	0.25	B.02	R.10	P.19		
2c	dark purple	0.39			P.25	R.31	
2d	blue	0.70	B.03		B.19	R.31	
3a	pink	0.10	R.03			R.32	
3b	pink	0.19	R.01			R.32	
3c	pink	0.27		R.10	B.19	R.32	
3d	blue	0.32				R.34	
3e	pink	0.53				R.33	
3f	blue	0.73	B.04			R.31	B.64
4a	pink	0.13	R.0			R.34	
4b	purple	0.33		R.12	P.23	R.33	
4c	pink	0.54				R.33	
Mean R_f of purple compound		0.38			0.23		
Tryptophan		0.37			0.24		
IAA		0.81			0.80		

R = red or pink

P = purple

B = blue

Table VIII. Colour reactions with DMAC and mean R_f values in isopropanol: ammonium hydroxide (28%):water (8:1:1) of regions of Phaseolus multiflorus extracts initially developed in isobutanol: methanol:water (80:5:15)

Extract identification and data from fig 4			Colour reaction with DMAC and R_f value in isopropanol:ammonia: water (8:1:1)					
Extract	Colour with DMAC	R_f region in but:meth:H_2O						
1a	red	0-0.05	R.06	B.12	R.15			
1b	pink	0.07	R.06		R.15			
1c	purple	0.15				P.27		R.39
2a	red-purple	0	R.03	B.10				R.40
2b	purple	0.13	R.05			P.27		
3a	blue	0.09	R.05			BP.23		R.43
3b	pink	0.24						R.43
4a	pink	0	R.03	B.10		B.26		R.43
4b	purple	0.10	R.05			P.26		R.43
4c	pink	0.25					B.36	R.43
Mean R_f of purple compound		0.13				0.27		
Tryptophan		0.12				0.28		
IAA		0.63				0.40		

R = red or pink

P = purple

B = blue

Table IX. Colour reactions with DMAC and mean R_f values in isopropanol: ammonium hydroxide (28%):water (8:1:1) of regions of chromatograms of Phaseolus multiflorus extracts initially chromatographed in butanol:ethanol (95%):water (4:1:1)

Extract identification and data from fig 5			Colour reaction with DMAC and R_f value in isopropanol:ammonia: water (8:1:1)				
Extract	Colour with DMAC	R_f region in but:eth:H_2O					
1a	blue	0.07	R.05	B.09	P.33		
1b	pink	0.12	R.07		P.33		R.43
1c	purple	0.19			P.29		R.44
2a	blue purple	0.10	R.04		P.27		R.36
2b	purple	0.21	R.06		P.27		R.42
3a	pink	0.11	R.05		B.23		R.44
3b	purple	0.16	R.05		B.23		R.45
3c	pink	0.31					R.45
4a	pink	0.10	R.04		B.25	B.35	R.42
4b	purple	0.18	RP.05		P.24		R.42
4c	pink	0.30		B.05	B.22		R.40
Mean R_f of purple compound		0.18			0.26		
Tryptophan		0.19			0.27		
IAA		0.77			0.35		

R = red or pink

P = purple

B = blue

Table X. Summary of R_f values of three endogenous indole compounds extracted with methanol from Phaseolus multiflorus seedling tissue chromatographed in different solvents

Colour with DMAC	R_f in			
	PAW	PAcW	BMW	BEW
Pink	0-.09	.06	0-.05	.03
Blue purple	.10	.70	0	.04
Purple	.28 (.23-.33)	.33 (.20-.43)	.12 (.09-.15)	.19 (.14-.25)

PAW isopropanol:ammonium hydroxide (28%):water (8:1:1)

PAcW isopropanol:acetic acid:water (4:1:1)

BMW isobutanol:methanol:water (80:5:15)

BEW n-butanol:ethanol (95%):water (4:1:1)

As a further check on the compound suspected to be tryptophan, extracts of shoots and cotyledons of Runner bean seedlings were chromatographed separately and the regions of the chromatograms corresponding to the purple band with DMAC were extracted four times with methanol. The extracts were evaporated to dryness under reduced pressure at 40° C and then redissolved in citrate sample diluting buffer, pH 2.2. One ml samples were analyzed by column chromatography using a Spinco Amino Acid Analyzer. Samples of a standard tryptophan solution were run separately.

The compound extracted from cotyledons corresponded in elution time and shape of curve to that obtained with the standard tryptophan solution. The shoot extracts contained some impurities

which interfered with resolution of the tryptophan peak. As a further check known quantities of L-tryptophan and C^{14}-labelled tryptophan were added to the shoot and cotyledon extracts and fractions were collected after passing through the amino acid analyzer. The synthetic tryptophan exactly superimposed on the peak corresponding to the endogenous compound and the amount the peak was increased corresponded to the amount of synthetic tryptophan added (0.25 mmole/ml). One ml samples of the eluate (which was collected at 1 min. intervals) were plated out and the radioactivity measured using a Gas Flow counter. The radioactivity in the eluate corresponded exactly to the peak obtained on the Analyzer recorder. (In addition to the main tryptophan peak there were other sharp peaks which eluted ahead of the tryptophan. A radioactively-labelled compound was also found which corresponded to one of these other compounds. The activity in this region was less than a tenth of that found in the tryptophan peak.)

On the basis of the foregoing tests the compound under investigation corresponds to tryptophan. From the amino acid analyzer readings the concentration of tryptophan extracted from cotyledons is 0.359 μmoles or 73 μg/g fresh weight. A quantitative measurement of that extracted from shoots was not possible because of interference of unknown substances, though it would probably be of the same order.

Chromatograms of plant extracts developed in isopropanol: ammonium hydroxide (28%):water (8:1:1) and isopropanol:acetic acid: water (4:1:1) were also sprayed with ninhydrin. The region corresponding to the purple compound with DMAC reacted with the ninhydrin as did several other bands.

Summary and Discussion

Using the method of Fletcher and Zalik for the estimation of IAA in seedlings, no IAA was able to be detected in seedlings of Phaseolus vulgaris (var. Dutch brown beans) or Phaseolus multiflorus (var. Scarlet Runner beans). However, a different batch of seeds of Phaseolus vulgaris from that used by Fletcher and Zalik was used in the present study. Compared with the previous batch these were lighter brown in colour and it is possible that they differed also in IAA content. However, the evidence obtained in the present study suggests that the compound measured by Fletcher and Zalik could have been tryptophan and not IAA. This suggestion is derived from the following facts. IAA and tryptophan have R_f values near to each other in the chromatography solvent used for the separation of the plant extracts. R_f values vary considerably from one run to another and the R_f regions of IAA and tryptophan may overlap in different runs. R_f values given by Fletcher and Zalik for IAA were obtained for tryptophan in several instances in the present study. The colour reaction with DMAC is the same for both IAA and tryptophan. The characteristics of the u.v. absorption spectra for IAA, tryptophan and the indole compound extracted from plant tissues are essentially identical except for slight differences in the minimum absorbance between 240 and 245 mμ. Although several variations in the technique were tried, in no case was a compound corresponding to IAA obtained. The presence of tryptophan was not noted by Fletcher and Zalik, although it probably would have been reported if it were present because of the high concentrations and the fact that it could easily be confused with IAA.

However, Fletcher and Zalik (1964) found a good correlation between estimations of IAA based on the spectrophotometric method and bioassay. The value of bioassays on chromatograms of crude extracts, however, is questionable because of the large number of other compounds present, some of which, e.g. sugars, organic acids, potassium ions, also have growth promotion properties (Thimann, 1963a).

The indole compound extracted from plant material was identical to tryptophan in its R_f values in 4 chromatography solvents, and its colour reaction with DMAC. Its elution time on column chromatography using a Spinco Amino Acid Analyzer was also identical with that of tryptophan. The amount of tryptophan present in cotyledons of Runner bean seedlings, as determined using the amino acid analyzer was 73 µg/g fresh weight. Although in table III a standard curve for IAA was used in calculating concentrations of the indole compound from spectrophotometer readings, the figure obtained for tryptophan content of cotyledons, correcting for the difference in molecular weight between IAA and tryptophan, is 65 µg/g fresh weight. This calculation presumes that the molar extinction coefficients for IAA and tryptophan are the same. This value, however, is in close agreement with the value (73 µg/g) obtained using the amino acid analyzer, and is similar to the value obtained by Lawrence and Grant (1963) for pea seedling cotyledons.

Although several other indole compounds were detected in plant extracts, these were not identified. These compounds as well as tryptophan were obtained by diffusion upwards from excised epicotyls of both Runner bean and Dutch brown bean seedlings, and also downwards from epicotyls of Runner bean seedlings.

SECTION B. INJECTION EXPERIMENTS

Fletcher and Zalik (1964) found that when IAA-C^{14} was injected into cotyledons of Phaseolus vulgaris (var. Dutch brown beans) label was translocated up into the epicotyl and young leaves as well as downwards. In this study the translocation of label into shoots was investigated further to see whether it was in fact IAA-C^{14} itself or a metabolite of this compound that was being translocated, and in addition an attempt was made to determine in what tissue the translocation took place.

In preliminary experiments it was found that the transport of label upwards also occurred in P. multiflorus (var. Scarlet Runner beans) and Zea mays. These were more convenient plants in which to study this phenomenon because of their hypogeal germination.

Injection of Phaseolus multiflorus Seedlings with IAA-C^{14}

Five µl of a solution of IAA-C^{14} in ethanol were injected into each cotyledon of eight 5-day old dark-grown Runner bean seedlings. The concentration used for injection was 0.2 µc/10 µl (2.6 µg/10 µl). Plants were left in the dark for 1, 3, 7 and 24 hrs, at which times duplicate plants were removed, divided into shoots, cotyledons and roots and these parts extracted for 2½ hrs at 3° C. The methanol extracts were then filtered, evaporated to small volumes and transferred quantitatively to planchets, dried and counted. The results given in table XI were obtained for shoots.

Table XI. Radioactivity extracted at various time intervals from shoots of Phaseolus multiflorus seedlings following injection of a solution of IAA-C^{14} in ethanol into the cotyledons

Time after injection (hr)	cpm/shoot
1	93
3	1280
7	1550
24	1560

Thus label was translocated quickly into the shoots. Chromatography of similarly prepared extracts revealed that the label stayed mainly near the origin with very little, or none in the case of the shoot extract, at an R_f value corresponding to IAA-C^{14}.

It was noticed when the cotyledons were being sliced before grinding that where the solution was discharged into the cotyledons there was a hole and that the tissue surrounding this was whiter than the rest of the tissue of the cotyledon. Since this also occurred in the controls which were injected with the same volume of ethanol, the damage was attributed to the ethanol. Since it was convenient to use ethanol as the solvent for IAA, the concentration of ethanol which could be tolerated by the tissue was investigated. Various concentrations of ethanol in water were injected into Runner bean cotyledons in the usual manner, and after 28 hrs the cotyledons were sliced and the sections examined for damage. It was found that a concentration of

25% ethanol or less injected in 5 or 10 µl quantities per cotyledon caused no observable physical damage though concentrations greater than this caused definite damage. Subsequently 10% or 20% alcoholic solutions were used for injection to avoid any artifact caused by killing of or damage to the tissue.

The injection experiments with Runner bean seedlings were repeated using IAA-C^{14} in 10% ethanol, 10 µl being injected per cotyledon. The dose used was 0.1 µc or 1.3 µg/plant. Duplicate shoots were extracted with methanol after various times and the results given in table XII were obtained.

Table XII. Radioactivity extracted at various time intervals from shoots of Phaseolus multiflorus seedlings following injection of a solution of IAA-C^{14} in 10% ethanol into the cotyledons

Time after injection (hr)	cpm/shoot
1	54
3	220
6	130
9	520
24	480
72	2840

Again label moved quickly into the shoots. Chromatography of these extracts showed that a labelled compound with an R_f value of IAA was

extractable from the shoots but most of the activity stayed near the origin (R_f values 0, 0.05, 0.07).

Injection of Zea mays Seedlings with IAA-C^{14}

Approximately 10 µl of a solution of IAA-C^{14} in 10% ethanol, containing 0.65 µg was injected into the endosperms of 3-day old dark-grown seedlings of corn (var. Morden). After 8 hrs. the seedlings were cut below the nodes, agar blocks applied to the cut surfaces and kept in a humid atmosphere. These were removed after 2½ hrs. and transferred to methanol. The internodes were re-cut and fresh agar blocks applied for a further period of 3 hrs. These were pooled with the previous blocks in methanol. The blocks were extracted overnight at 3° C, the methanol decanted and the blocks re-extracted twice with fresh methanol for periods of approximately 3 hrs. The extracts were pooled.

The apical 6 - 10 mm of the coleoptiles were excised and placed on agar blocks in covered Petri dishes for 5½ hrs. The agar blocks were extracted with methanol. The coleoptile tips, and the pieces of the shoots removed between the internodes and the coleoptile tips (i.e. remains of the internodes, nodes, remains of the coleoptiles and also the first leaves) were crushed and extracted with methanol.

Extracts were chromatographed, and radioautograms made of the chromatograms.

In all four extracts distinct bands on the radioautograms corresponding to IAA-C^{14} were detected. In the extracts of the tissues,

traces of compounds with low R_f values were also detected. Chromatograms of methanol extracts of seeds which had been injected with IAA-C^{14} revealed major peaks at R_f values 0.37 - 0.46 (the R_f region of IAA) with minor peaks at 0.09 - 0.13.

Injection of Phaseolus multiflorus Seedlings with Tryptophan-C^{14}

Five µl of a solution containing 0.003 µmoles (0.61 µg) of tryptophan-C^{14} were also injected into cotyledons of Runner bean seedlings. Growing tips, epicotyls and cotyledons of 4 plants were extracted separately with methanol after 20 - 24 hrs and chromatographed in two solvents, (1) isopropanol:ammonium hydroxide (28%):water, 8:1:1, and (2) isopropanol:acetic acid:water, 4:1:1. In cotyledons the major methanol extractable labelled compound was tryptophan but another compound present in a smaller amount was also extracted. This compound had an R_f value of 0.13 in solvent 1 and 0.72 in solvent 2. In epicotyls the second compound was again present but tryptophan was present in a greater amount. In the extract of the growing tips again both tryptophan and the other compound were present with the other compound being present in a slightly larger amount than tryptophan.

As Thimann (1948) and Leopold (1961) point out, if IAA unnaturally enters the transpiration stream then it will be carried upwards with it. In view of this the question whether in these studies the IAA was being injected directly into the transpiration stream and thereby transported upwards was raised. To answer this it would be important to know whether vascular tissue was present in the structures into which the solutions were injected, and if so the extent of it.

Little recent information could be obtained on this subject. Esau (1960) mentions vascular traces leaving the hypocotyl to enter the cotyledons. Eames and MacDaniels (1947) mention that in thick cotyledons that do not expand on germination the vascular system is much simplified. Somewhat vague descriptions were given by Godfrin (1884) and Pammel (1899) of vascular tissue in cotyledons of Phaseolus sp. but the extent of it was not described. It was therefore necessary to examine these structures microscopically.

Anatomical Study of Cotyledons and Endosperms

Approximately 3 - 4 mm slices of cotyledons of 6-day old seedlings of P. vulgaris and P. multiflorus and endosperms and scutella of 3-day old seedlings of Zea mays were fixed in formalin-acetic acid-alcohol. After fixation the segments were embedded in Tissuemat according to standard procedures (Johansen, 1940).

Sections (15 - 20 μ thick) were satisfactorily obtained of cotyledonary tissue although difficulty was encountered in preparing sections of corn endosperms. All sections were stained with safranin and fast green.

Microscopic examination of cotyledons revealed an extensive vascular system. Bundles containing both phloem and xylem branched generally throughout the cotyledons running mainly longitudinally from the region of attachment to the stem (figs 6-9). Bundles were generally evenly spaced on the outer side of the cotyledon; other bundles were more randomly spaced. Associated around the vascular bundles were cells containing many inclusions (mainly starch grains). Cells further from the vascular bundles were generally devoid of these inclusions.

FIG. 6. Transverse section of cotyledon of Phaseolus multiflorus showing vascular bundles and cells containing inclusions distributed around the vascular bundles. (x 70)

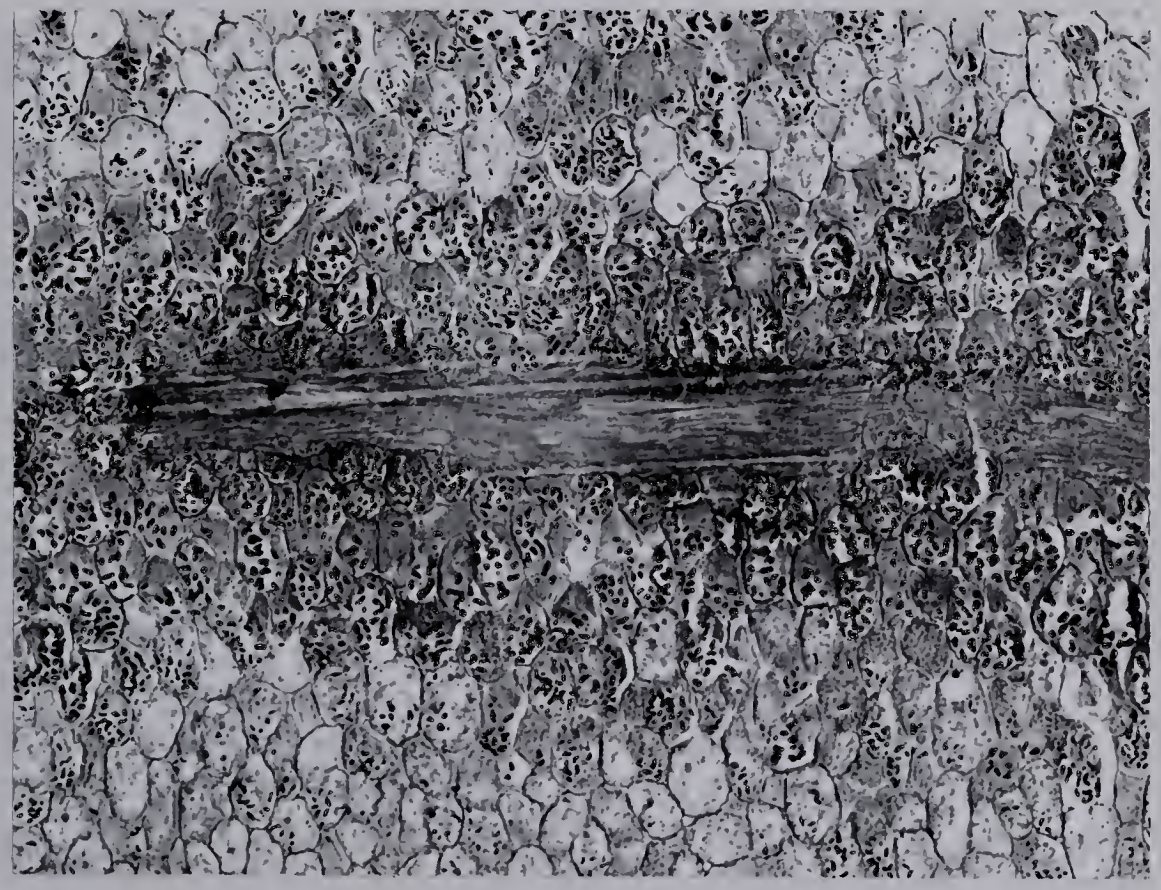


FIG. 7. Longitudinal section of cotyledon of Phaseolus multiflorus showing vascular bundles and cells containing inclusions distributed around the vascular bundles. (x 70)

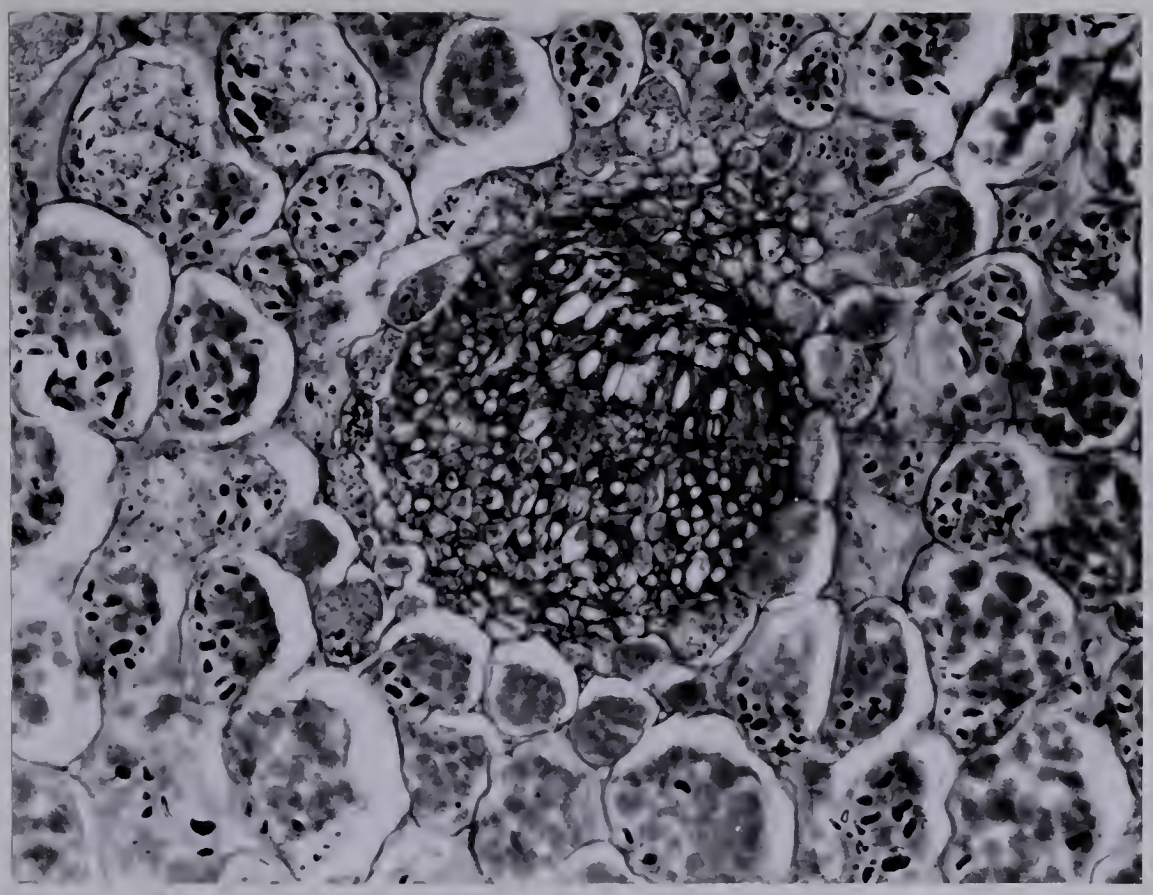


FIG. 8. Transverse section of cotyledon of Phaseolus multiflorus showing vascular bundle. (x 230)

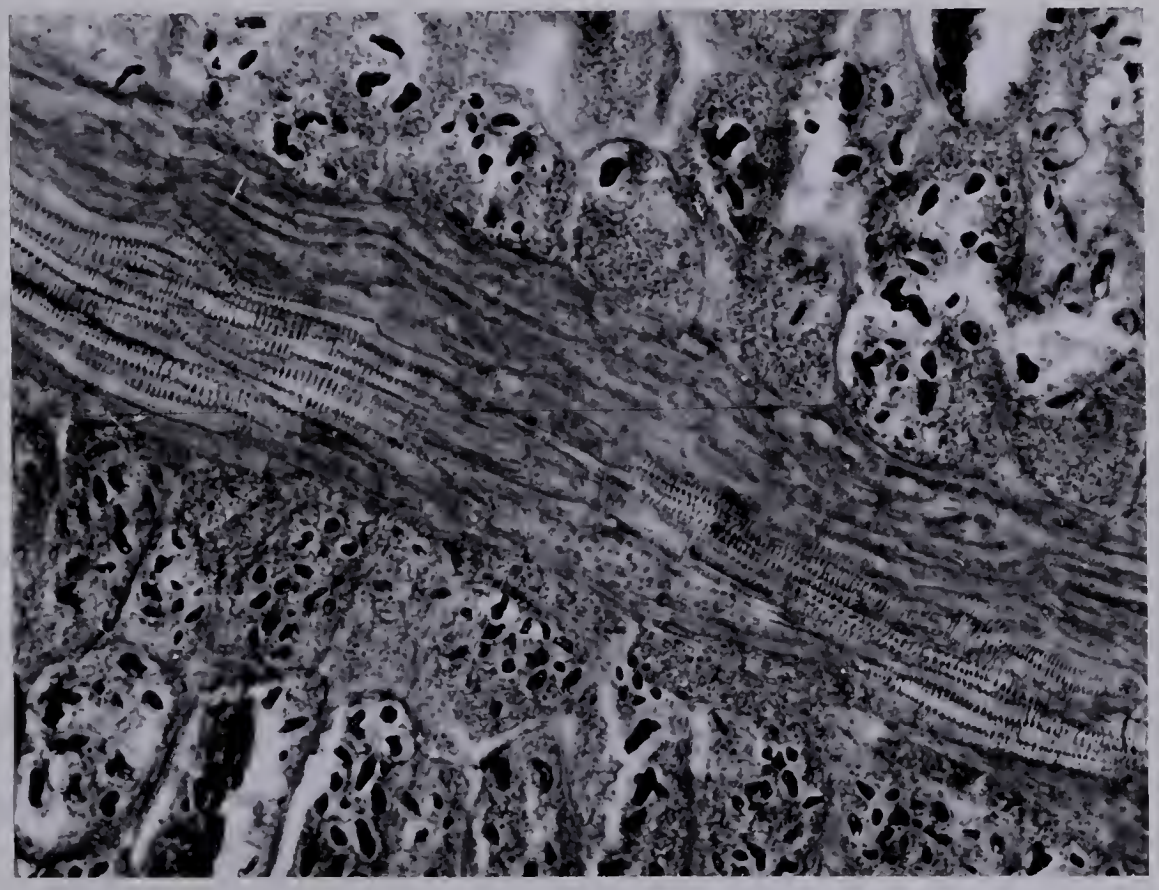


FIG. 9. Longitudinal section of cotyledon of Phaseolus multiflorus showing vascular bundle. (x 230)

Examination of corn endosperm indicated that it was devoid of vascular tissue but that a definite arrangement of vascular tissue was present in the scutellum.

Vital Staining of the Transpiration Stream in Seedlings

Further studies to demonstrate the transpiration stream employed the technique of vital staining (Johansen, 1940). The two stains used were basic Fuchsin and Light Green SF Yellowish. Roots of seedlings were placed in the vital stain and some of the roots were cut to allow easier penetration. Basic fuchsin proved unsatisfactory as it was taken up slowly and irregularly. Light green (Harvey, 1930), however, was taken up quickly and uniformly. The extensive vascular system of Runner bean cotyledons could be clearly seen, especially when the cotyledons were sectioned (figs 10 and 11).

With corn seedlings no dye entered the endosperm but a distinct cylinder of vascular tissue was observed passing through the scutellum. This branched to enter the interface between the scutellum and endosperm. In the coleoptile the two vascular bundles became clearly stained. Sometimes a third column of stain was observed in the coleoptile but on sectioning such stained coleoptiles it was found that this third column was associated with the thin region in older coleoptiles where the coleoptile later splits to allow emergence of the leaves.

Because of the presence of vascular tissue in the cotyledons, it seemed possible that IAA-C^{14} was translocated to the shoot by gaining artificial entrance into the xylem through injection. To test this

FIG. 10. Transverse sections of a cotyledon of Phaseolus multiflorus after allowing the dye, Light Green, to be taken up through the cut roots. (x 6)

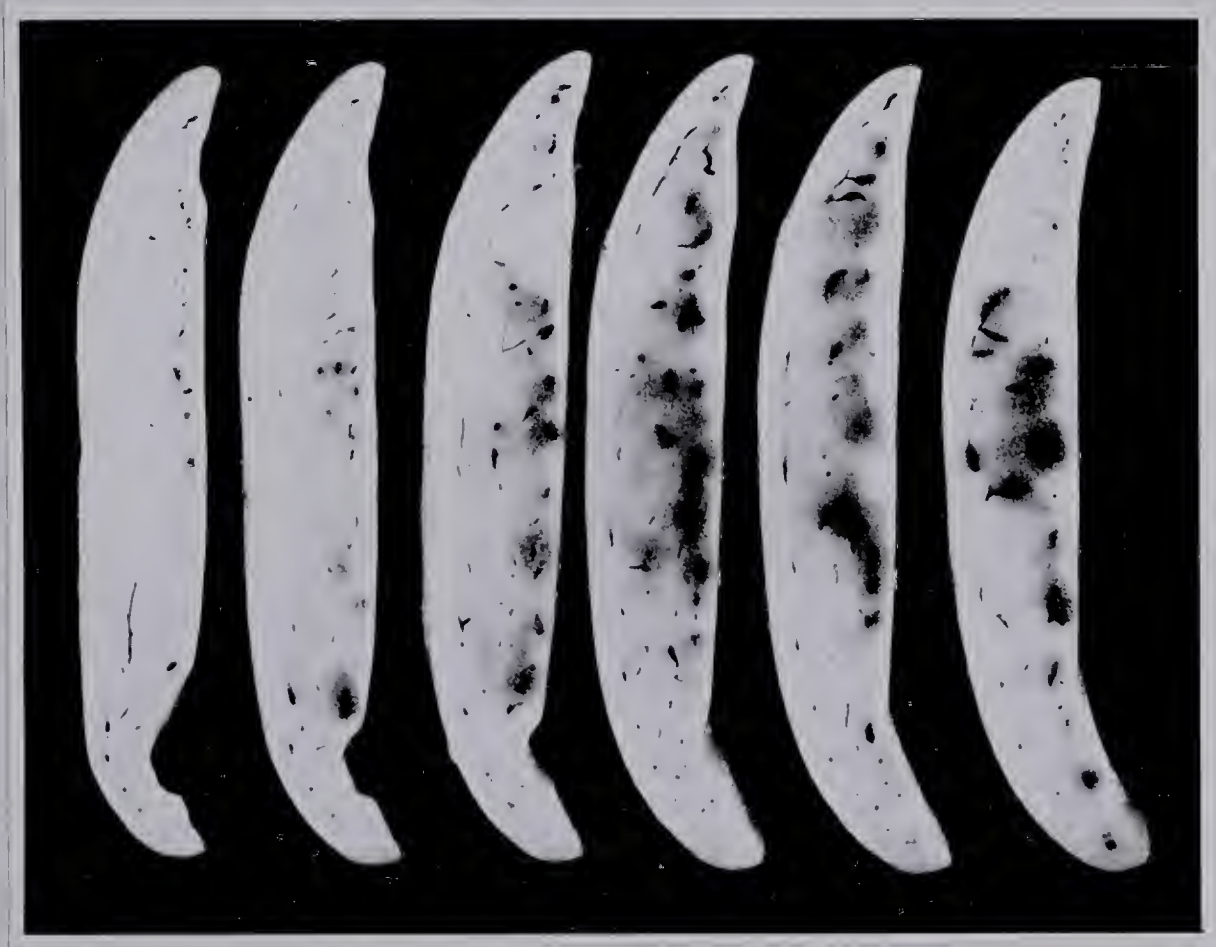


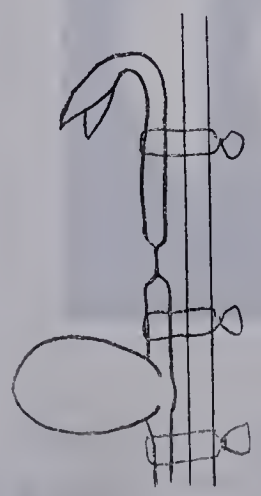
FIG. 11. Longitudinal sections of a cotyledon of Phaseolus multiflorus after allowing the dye, Light Green, to be taken up through the cut roots. (x 4)

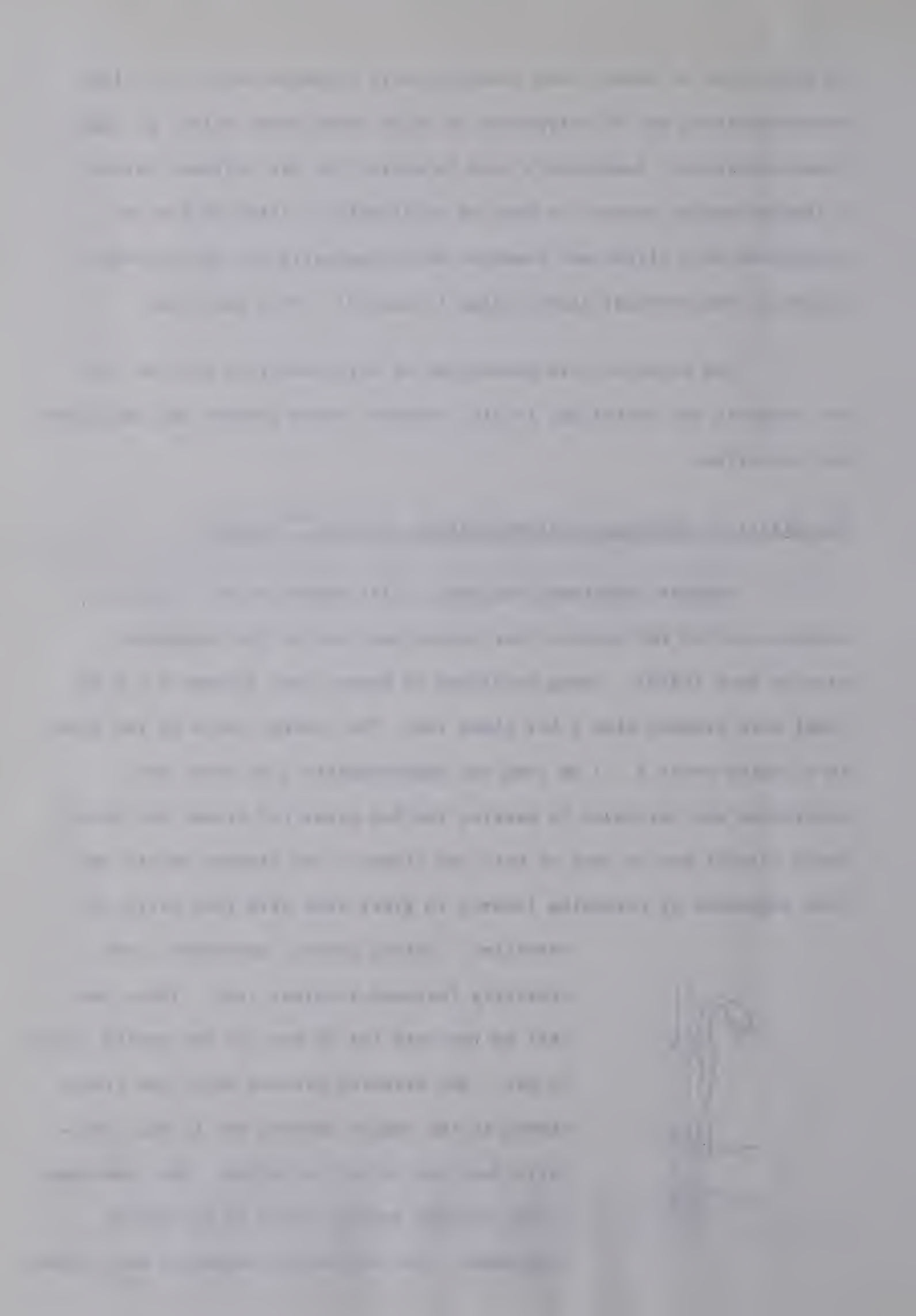
20 cotyledons of Runner bean seedlings were injected with 10 µl Light Green/cotyledon and 20 cotyledons of Dutch brown beans with 5 µl Light Green/cotyledon. Immediately upon injection the dye diffused through a limited region around the zone of application. After 48 hrs the cotyledons were sliced and examined microscopically for any evidence of dye in the vascular tissue (figs 12 and 13). None was found.

Dye injected into endosperms of corn seedlings did not reach the xylem in the scutellum; it did, however, enter between the endosperm and scutellum.

The Effect of Branding on Translocation of IAA-C^{14} and Dye

Another experiment designed to illustrate whether IAA was translocated in the xylem to the shoots made use of the technique used by Snow (1929). Young seedlings of Runner bean (shoots 5 - 8 cm long) were branded with a hot glass rod. The living tissue of the shoot in a region about 6 - 7 mm long and approximately 3 cm above the cotyledons was destroyed by passing the hot glass rod around the shoot, being careful not to char or boil the tissue. The branded shoots were then supported by fastening loosely to glass rods with thin strips of Parafilm. Control shoots, unbranded, were similarly fastened to glass rods. These were left in the dark for 24 hrs for the heated tissue to die. The branding process kills the living tissue in the region treated but if done carefully does not affect the xylem. The appearance of the branded regions after 24 hrs was as diagrammed. The following treatments were given:





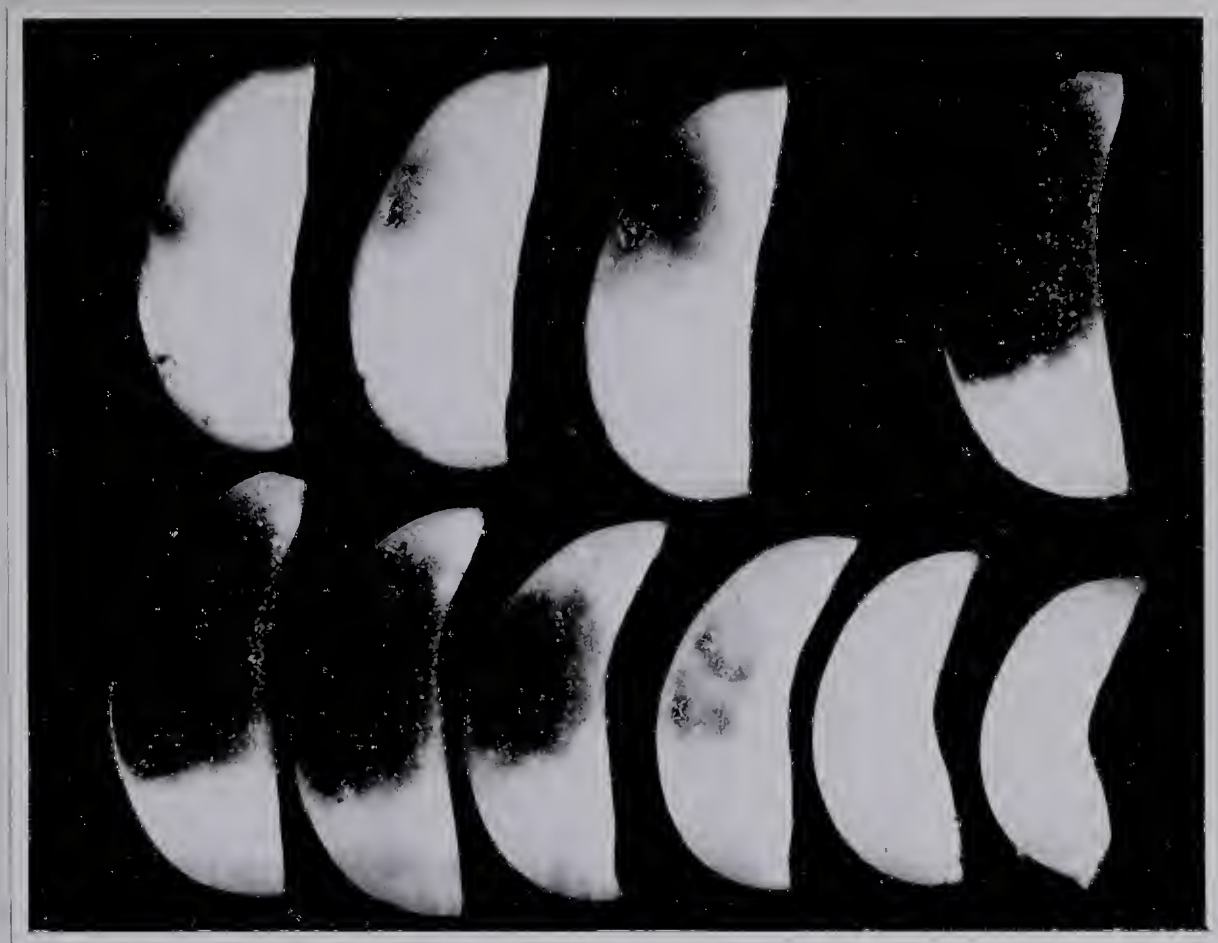


FIG. 12. Transverse sections of a cotyledon of Phaseolus multiflorus after injection with the dye, Light Green. (x 6)

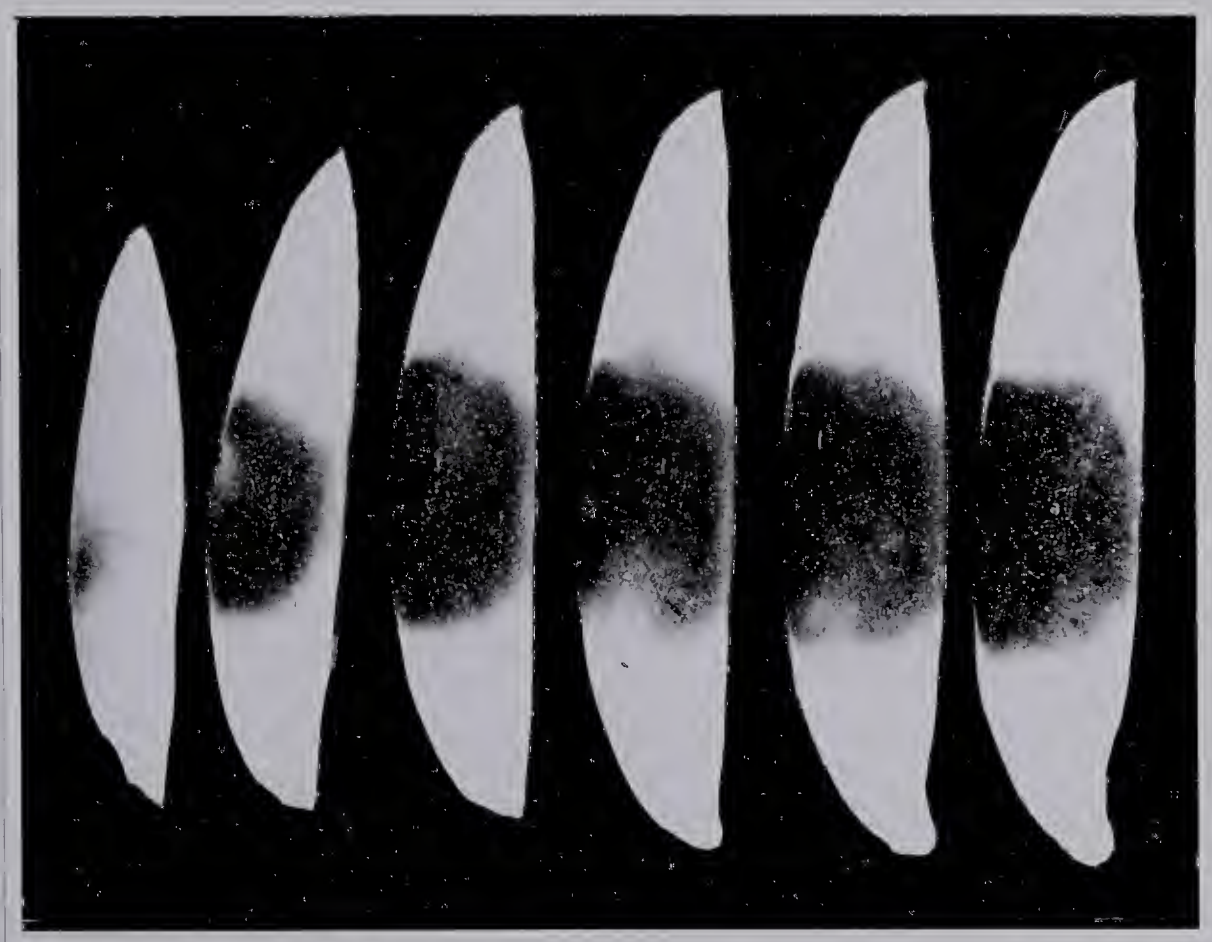


FIG. 13. Longitudinal sections of a cotyledon of Phaseolus multiflorus after injection with the dye, Light Green. (x 4)

(a) Branded plants injected with IAA-C^{14} and kept in dark.

(b) Branded plants injected with IAA-C^{14} and kept in light.

(c) Branded plants, roots cut and placed in Light Green dye and kept in dark.

(d) Branded plants, roots cut and placed in Light Green dye and kept in light.

(e) Unbranded plant injected with IAA-C^{14} and kept in dark.

(f) Unbranded plant injected with IAA-C^{14} and kept in light.

(g) Unbranded plants, roots cut and placed in Light Green dye and kept in dark.

(h) Unbranded plants, roots cut and placed in Light Green dye and kept in light.

A fan was placed in the dark room and directed towards the plants to increase transpiration. Those kept in the light were placed near a north facing window.

After 24 hrs shoots of the plants of which the cotyledons were injected with IAA-C^{14} were cut about 5 mm above the dead region of the brand and at a corresponding height on the unbranded shoots. These shoots were ground and extracted with methanol for 3 hrs. The extracts were plated out on planchets and counted. The results obtained are given in table XIII.

Table XIII. Radioactivity extracted from branded and unbranded shoots of Phaseolus multiflorus seedlings following injection of IAA-C^{14} into the cotyledons

Treatment	cpm/shoot
Branded, in dark	8
Branded, in light	5
Unbranded, in dark	386
Unbranded, in light	550

These results show the marked difference in the amounts of label translocated through the branded and unbranded shoots, that in the branded shoots being only just above background.

Examination of the dye-treated plants revealed that the dye had been translocated into the shoots of all plants, indicating that the xylem strands remained intact in the branded plants. The branded shoots in all cases had grown very little whereas the unbranded shoots had grown considerably during the 24 hr period. These experiments were repeated with similar results.

These results indicate that the label was not translocated into the shoot by the xylem, but by living tissue.

Summary and Discussion

When small quantities of IAA-C^{14} were injected into cotyledons of Runner bean seedlings or endosperms of corn seedlings, label was found to move up into the shoots in a short time. On chromatography of

extracts of Runner bean shoots so treated, a labelled compound with an R_f of IAA was detected in addition to radioactivity which stayed near the origin. In corn seedlings the acropetal movement of a labelled compound with an R_f of IAA was shown by collection of the diffusate from cut internodes. This was the only labelled compound collected. A similar compound was obtained diffusing downwards from coleoptiles of treated plants. In extracts of the coleoptile tips and internodes, this compound was also detected in addition to traces of 1 or 2 compounds with low R_f values.

Since IAA-C^{14} was administered and the major compound detected was at the same R_f region as IAA, further identification was not carried out, but the compound was presumed to be IAA-C^{14}. In view of the basipetal polarity of IAA transport in seedling shoots, this acropetal movement of IAA-C^{14} was surprising.

The presence of extensive vascular systems in cotyledons of Runner bean and Dutch brown bean has been shown. Endosperms of corn were shown to be lacking in vascular tissue.

In view of the presence of vascular tissue in cotyledons and the fact that IAA will be translocated in the xylem if it unnaturally gains access to it (Thimann, 1948; Leopold, 1961) the possibility of direct injection of dye into the xylem was considered. However, the vital stain, Light Green, injected into cotyledons or endosperms in high concentrations did not gain access to the transpiration stream. Although the dye differs from IAA in not being a naturally occurring compound in plants and having a higher molecular weight than IAA, if IAA

moved to the shoot through being injected directly into xylem vessels, then so should the dye. Dye has been shown to move readily through the transpiration stream by administering it through cut roots. However, IAA could have gained entrance into the xylem not by direct injection but by being in a high local concentration around the xylem vessels, for IAA has also been shown to travel in the transpiration stream if the plant is treated with a higher than physiological amount (see Thimann, 1948; Leopold, 1961). Although only small amounts of IAA-C^{14} were injected (0.65 to 2.6 μg) the physiological concentrations present in the plant tissue was not determined. The amounts used, however, would probably not be much in excess of the physiological levels.

From the vital staining experiments it was demonstrated that xylem is continuous from the roots to the cotyledons, i.e. that the xylem in the cotyledons conducts away from the stem to the distal end of the cotyledons. However, whether a xylem system also exists which conducts from the cotyledons up into the epicotyl has not been shown. That such a system exists, in fact, seems unlikely if one thinks teleologically, for there appears to be no reason why the transpiration stream should take a detour through the cotyledons, unless it plays a relatively large part in the conduction of organic nutrients from the storage tissue to the shoot. Such a system could also, of course, be an evolutionary left-over.

Based upon the experiments in which living tissue was destroyed in a region of the epicotyl but the xylem left intact, as evidenced by the ability of such plants to translocate dye from the roots to the

epicotyl, it seems unlikely that the IAA-C^{14} was translocated in the xylem. The fact that label was not translocated acropetally in branded plants indicates that the acropetal transport took place only in living tissue.

SECTION C. TRANSLOCATION EXPERIMENTS

Polarity Studies

It was found in Section B that IAA-C^{14} injected into cotyledons and endosperms was translocated acropetally in the shoots. Therefore, the purpose of the translocation experiments was to investigate the strength of polar transport of IAA in epicotyls of Phaseolus multiflorus and coleoptiles of Zea mays. In addition an attempt was made to ascertain the tissue through which IAA is translocated in the polar manner.

Tryptophan was found to be present in relatively large amounts in Runner bean seedlings, and it was also found to be the major indole compound collected in agar blocks applied to cut surfaces of Runner bean epicotyls (Section A). Tests were conducted therefore to determine if there was any transport polarity for this compound.

Methods

Studies on polarity of transport through tissue segments were similar to those described by Leopold (1955), Goldsmith and Thimann (1962), McCready and Jacobs (1963), Black and Osborne (1965).

Six- to seven-day old dark-grown Runner bean seedlings 10 cm tall were used. Segments of epicotyl 6 mm in length were removed from 1 cm below the hook with a guillotine.

Corn seedlings, 3.5 - 4 cm tall, were used for translocation studies on the third day after sowing. Coleoptiles were excised just above the node with a razor blade so that only the coleoptiles and not

the first leaves inside were cut. The coleoptiles were removed from the first leaves, and using a guillotine a 5.1 mm segment was cut out 2 mm below the tip.

The segments of both Runner bean and corn were handled with forceps on the points of which were fastened extensions of thin cardboard so that even when the forceps were fully closed the tissue held between the tips of the cardboard extensions was not damaged by pressure.

Both Runner bean and corn seedlings were kept in darkness except as mentioned in general materials and methods. Manipulations were carried out using the green safelight.

Cylindrical agar blocks were used instead of the usual agar blocks which are square in cross-section. Bacto Agar was dissolved in distilled water by boiling. The final agar concentration for all blocks was 1.5%. For blocks containing additives (e.g. radioactively-labelled IAA or tryptophan) the measured solution and the agar solution were mixed in a glass vial kept in a water bath at 45^{o} - 55^{o} C using a previously warmed 1 ml glass syringe. The agar was generally initially hotter than the water bath temperature though rapid cooling occurred because of the small volumes used. After thorough mixing the labelled agar was transferred with the syringe to a glass tube which was stoppered at the lower end and kept in the water bath to maintain the agar molten. The agar was allowed to run slowly down the tube and fill up without forming bubbles. The tube of agar was then removed from the water bath and allowed to solidify either at room

temperature or by placing in cold water. Tubes of 4 mm internal diameter were initially used for both corn coleoptiles and Runner bean epicotyls, but later 3 mm blocks were used for corn coleoptiles as the 4 mm blocks were unnecessarily large. When the agar had solidified the stopper was removed and the rod of agar extruded onto a glass plate. The rod was then sliced into 2.0 mm blocks with a guillotine. The volumes of the 4 mm diameter blocks were 25 mm^3 and of the 3 mm blocks, 14 mm^3.

Ten replicates were used for each treatment. The agar blocks and tissue segments were arranged in covered Petri dishes to prevent dehydration. To reduce further any possible drying out the Petri dishes were kept in a humidified cabinet. The temperature of the atmosphere in the cabinet was 20° C $\pm$ 1° C.

After diffusion times of 3 or 6 hrs the agar blocks were removed, and placed in the centre of aluminum planchets to which a small amount of Haupt's adhesive had been added. These were dried in the air and counted using a Gas Flow Counter. Since all samples were treated and counted in the same manner, no allowance was made for self-absorption.

In some cases the agar blocks and tissue segments were extracted with methanol after the translocation experiment. These extracts were chromatographed, scanned, and radioautograms made.

Results

The treatments using IAA-C^{14} with corn coleoptiles and Runner bean epicotyls are diagrammed and tabulated in tables XIV and XV. From the results presented in these tables it can be seen that there is a strict basipetal polarity of IAA-C^{14} translocation in both corn coleoptiles and Runner bean epicotyls. No activity was found to be translocated in an acropetal manner. With the corn coleoptile segments and 10^{-4}M IAA-C^{14} doubling the diffusion time approximately doubled the amount translocated through to receiver blocks (table XIV). With tryptophan-C^{14} very little or none was translocated through to the receiver blocks (tables XVI and XVII). In all cases label was taken up from the donor blocks by the tissue. It is interesting that in every experiment the treatment in which least label was taken up from the donor blocks was treatment C, with tissue in normal orientation arranged to test acropetal translocation.

After counting the agar blocks for treatment A with tryptophan-C^{14} on Runner bean epicotyls, they were extracted with methanol and chromatographed. The fresh tissue segments were also extracted and chromatographed. Scanning of chromatograms of the receiver blocks showed the presence of a single labelled compound with an R_f value of tryptophan. This compound was barely detectable in the 3 hr treatment but was distinct in the 6 hr treatment. Actigraphs of chromatograms of the tissue extracts showed large peaks at R_f regions corresponding to tryptophan, and minor peaks at R_f values 0 and 0.08 - 0.14 for the 3 hr treatment. These peaks were considerably larger in the 6 hr treatment and also two more minor peaks with R_f values 0.66 - 0.69

Table XIV. Translocation of IAA-C^{14} in coleoptile segments of Zea mays. Results are averages of 10 replicates.

Orientation of tissue	Direction of transport	Time (hrs)	Final cpm in donor blocks	cpm in receiver blocks	% supplied activity in receiver blocks
Donor concentration 10^{-4}M (3950 ± 11* cpm)					
A normal	basipetal	3	3320 ± 10	290 ± 3.1	6.72
D inverted	acropetal	3	3610 ± 11	1 ± 0.2	0.02
A normal	basipetal	6	2370 ± 8.8	650 ± 4.6	16.5
D inverted	acropetal	6	3260 ± 10	0	0

* Standard deviations of counting rates.

A basipetal translocation, tissue normally orientated with respect to gravity.

B basipetal translocation, tissue inverted

C acropetal translocation, tissue normally orientated with respect to gravity.

D acropetal translocation, tissue inverted.

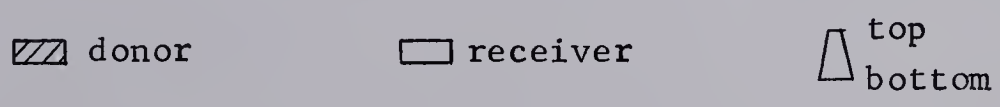


Table XV. Translocation of IAA-C^{14} in epicotyl segments of Phaseolus multiflorus. Translocation time 3 hrs. Results are averages of 10 replicates.

Orientation of tissue	Direction of transport	Final cpm in donor blocks	cpm in receiver blocks	% supplied activity in receiver blocks
Donor concentration 10^{-5}M (610 ± 6.3* cpm)				
A normal	basipetal	195 ± 2.6	26 ± 0.9	4.3
B inverted	basipetal	300 ± 3.1	20 ± 0.8	3.3
C normal	acropetal	355 ± 3.5	0	0
D inverted	acropetal	300 ± 3.1	0	0
Donor concentration 10^{-4}M (5790 ± 15 cpm)				
A normal	basipetal	2640 ± 9.2	245 ± 2.8	4.3
B inverted	basipetal	3570 ± 11	155 ± 2.2	2.7
C normal	acropetal	4690 ± 12	3 ± 0.3	0.05
D inverted	acropetal	3080 ± 10	4 ± 0.4	0.05

* Standard deviations of counting rates.

Table XVI. Translocation of tryptophan-C^{14} in coleoptile segments of Zea mays. Translocation time 3 hrs. Results are averages of 10 replicates.

Orientation of tissue	Direction of transport	Final cpm in donor blocks	cpm in receiver blocks	% supplied activity in receiver blocks
Donor concentration 10^{-5}M				
A normal	basipetal	2,710 ± 14*	7 ± 0.7	**
B inverted	basipetal	2,870 ± 15	5 ± 0.6	
C normal	acropetal	3,160 ± 15	4 ± 0.5	
D inverted	acropetal	2,990 ± 15	7 ± 0.7	
Donor concentration 10^{-4}M (14,380 ± 21 cpm)				
A normal	basipetal	10,200 ± 18	11 ± 0.6	0.08
B inverted	basipetal	11,550 ± 20	29 ± 1.0	0.20
C normal	acropetal	12,160 ± 20	5 ± 0.4	0.04
D inverted	acropetal	9,460 ± 18	7 ± 0.5	0.05

* Standard deviations of counting rates.

** These could not be calculated as counts in the original donor blocks were not obtained.

Table XVII. Translocation of tryptophan-C^{14} in epicotyl segments of Phaseolus multiflorus. Results are averages of 10 replicates.

Orientation of tissue	Direction of transport	Time (hrs)	Final cpm in donor blocks	cpm in receiver blocks	% supplied activity in receiver blocks
Donor concentration 10^{-5}M (2,510 ± 9.1* cpm)					
A normal	basipetal	3	2,110 ± 8.3	3 ± 0.3	0.12
B inverted	basipetal	3	2,070 ± 8.3	0	0
C normal	acropetal	3	2,300 ± 8.8	4 ± 0.4	0.16
D inverted	acropetal	3	1,920 ± 8.0	0	0
Donor concentration ca. 10^{-5}M (1,960 ± 8.0 cpm)					
A normal	basipetal	3	1,360 ± 6.8	0	0
B inverted	basipetal	3	1,280 ± 6.6	2 ± 0.3	0.10
C normal	acropetal	3	1,410 ± 6.8	3 ± 0.3	0.15
D inverted	acropetal	3	1,070 ± 6.0	4 ± 0.4	0.20
Donor concentration 10^{-4}M (16,800 ± 30 cpm)					
A normal	basipetal	3	14,450 ± 22	12 ± 0.6	0.07
B inverted	acropetal	3	13,730 ± 21	9 ± 0.5	0.05
A normal	basipetal	6	9,320 ± 18	39 ± 1.1	0.23
B inverted	acropetal	6	9,380 ± 18	13 ± 0.6	0.08

* Standard deviations of counting rates.

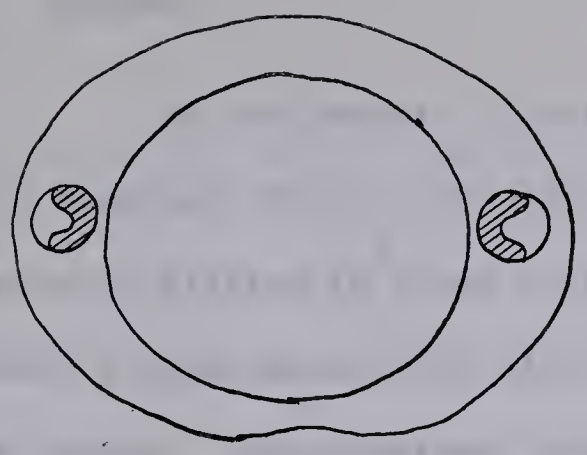
and 0.80 - 0.85 were present. The compounds at R_f values 0 and 0.08 were also present in the donor blocks, being in larger amounts after the 6 hr treatment than after the 3 hr treatment.

Only one labelled compound, corresponding in R_f value to IAA, was detected in receiver blocks after treatment of both corn and Runner bean tissue with IAA-C^{14}.

Translocation of Dye Through Tissue Segments

The usual arrangement for translocation experiments was set up using treatments A and D for basipetal and acropetal translocation. The non-toxic vital stain, Light Green SF Yellowish was used instead of label in the donor blocks.

With corn coleoptiles it was observed that, irrespective of whether the donor block was placed at the apical or basal end of the section, dye diffused into the sections to about 1 mm and then moved in two distinct columns only, which corresponded to the vascular bundles. Occasionally when a segment from an older coleoptile was used, a third track of dye was observed but on sectioning, this was found to be associated with the thin part of the coleoptile between the vascular bundles which later splits to allow the emergence of the leaves (c f . Section B). When free-hand sections were made of the coleoptiles it was found that in sections made after a few hours (up to 3 hrs) the dye was present only in the xylem (as diagrammed).



In sections left longer the dye was present in the rest of the vascular bundles though it was restricted to these structures. The dye was found to be translocated through the total length of the coleoptile segments (5.1 mm) in a few hrs. When excised coleoptiles about 1 - 1.5 cm long, with intact tips, were placed on blocks containing dye, it was translocated through the vascular bundles in a very short time and reached the tips in about 1 hour.

In Runner bean epicotyl segments also the dye was translocated mainly in the vascular tissue. At the end of the tissue distal to the donor block the dye was present almost exclusively in the xylem. Nearer to the donor the dye was also present in the phloem.

Tissue Autoradiography

Autoradiographic evidence of the tissue in which IAA is translocated in the polar manner has not been obtained. One of the difficulties involved is the fact that the polar transport mechanism is saturated at very low concentrations of IAA (Goldsmith and Thimann, 1962). Using radioactively-labelled IAA with a Specific Activity in the order of 10 mc/mM would result in sections with radioactivity very little above background. Tritium-labelled IAA with a Specific Activity of 112 mc/mM was available and the following experiments were designed in an attempt to show the tissue(s) involved in translocating this compound.

Methods

No preliminary translocation experiments were conducted using this compound since at the time no efficient means of detecting and measuring tritium (H^3) was available. But on the basis of the translocation experiments with IAA-C^{14} a donor concentration of 10^{-4}M IAA was chosen. Only treatment A was used (i.e. basipetal translocation with the tissue in normal orientation) for both corn coleoptiles and Runner bean epicotyls.

The following treatments were given:

A Diffusion time 1 hr.

B Diffusion time 3 hrs.

C Diffusion time 1 hour; then the donor block was replaced with an agar block containing the same concentration of cold IAA. Left for 2 hrs.

D Diffusion time 3 hrs, tissue laid horizontally.

E Tissue laid horizontally. Labelled agar blocks replaced after 1 hr with blocks containing cold IAA for 2 hrs.

F Epicotyl or internode left attached to seed and decapitated. Labelled agar block placed on cut surface and left 3 hrs.

G Same as F but labelled block replaced with block containing cold IAA after 1 hr.

Because IAA is soluble in water and alcohols, the usual procedures of fixing and embedding tissue could not be used. To prevent

leaching and in order to localize the radioactivity, it was necessary to freeze the segments and to section the tissue without its coming into contact with any solvent, and without being thawed.

Tissue segments were either flash frozen directly in isopentane cooled in dry ice or were first embedded in O.C.T. (Lab-Tek) pre-cooled to near 0° C, and then flash frozen. A small amount of the cooled O.C.T. was added to half a pharmaceutical gelatin capsule, the tissue placed into it, quickly orientated and the capsule dropped into the freezing mixture. Before freezing, the tissue was marked with dots of red ink at positions where they were to be sectioned, i.e. at about 1/4, 1/2 and 3/4 of the way along the segments. The frozen segments were transferred to a deep-freeze at -30° C and were later transferred packed in dry ice to a cryostat and maintained at -20° C. They were allowed to equilibrate to this temperature overnight and were then removed from the capsules and sectioned with a microtome contained in the cryostat.

Difficulty was experienced in sectioning the plant material especially corn coleoptiles. With or without the supporting material (O.C.T.) suitable sections of corn coleoptiles were not obtained. The tissue disintegrated upon sectioning. Better, though still poor, sections of Runner bean epicotyl 8 μ - ca. 25 μ thick were obtained. Using a camel's hair brush sections were transferred from the microtome knife to microscope slides. The slides had previously been treated with Haupt's solution and dried. Slides were placed in slide boxes which were transferred to pre-cooled flasks connected to the freeze-drying apparatus. The flasks containing the open slide boxes were immersed in an ice and salt

bath below -10° C to maintain the tissue frozen whilst being dried. The sections were thus dried for several hours. When dry, they were stored in slide boxes containing Drierite and sealed with tape.

Dipping the slides in Kodak NTB3 emulsion previously liquified by heating to 45° C and diluted 1:1 with distilled water, caused leaching of soluble materials from the sections. Another method of coating the slides with emulsion was therefore used. This was described by Miller et al. (1964). Thin films of emulsion were made by dipping loops made of nickel-chrome alloy wire into the melted emulsion and drying in the air at room temperature. Manipulations were carried out using a Kodak Wratten Series 2 red safelight with a 7½-watt bulb. Slides with sections mounted on them were passed slowly through the dried film on the loop and warm moist air (breath) directed onto the film to seal it to the slides. This small amount of moisture has been found not to dissolve or redistribute any soluble radioactivity in the sections (Miller et al., 1964). Difficulty was experienced in coating the sections with emulsion. The film tended to form bubbles over the sections. If this happened and was noticed, the bubble was touched with a needle to allow air to escape.

After 10 days some of the slides were developed and it was found that the O.C.T. itself had resulted in intense blackening of the emulsion. Other sections which were not embedded in O.C.T. were exposed for 3 months at room temperature in sealed boxes containing Drierite. These were developed in Kodak D-19 developer for 4 min, rinsed and fixed in Kodak fixer for 3 - 4 min.

Results

Macroscopic examination of the slides of Runner bean epicotyls showed that the zones of greatest blackening of the emulsion occurred in regions corresponding to the epidermis and cambial ring (figs 14 and 15). However, label was distributed generally throughout the sections. In some sections, particularly those taken from regions near the donor blocks, very dark spots corresponding to label in xylem patches were observed. Sections which had been administered label for 3 hrs were generally darker than those administered label for only 1 hr. Control slides were of sections prepared, coated with emulsion and exposed in exactly the same way as the treatments, but were fixed instead of being developed. In all treatments the sections were darker than the controls and background. Microscopic examination showed that the darkening was due to silver grains but did not provide any information not apparent from macroscopic examination.

Summary and Discussion

A strong basipetal polarity of transport of IAA-C^{14} was found in epicotyls of Runner bean seedlings at donor concentrations of 10^{-5} and 10^{-4}M and of coleoptiles of corn (var. Morden) at a donor concentration of 10^{-4}M. Table XV shows that inverting Runner bean epicotyl segments with respect to gravity caused a decrease in the amount of label translocated basipetally. A similar effect of gravity on basipetal translocation in corn coleoptiles was noted by Hertel and Leopold (1963). Although IAA-C^{14} was taken up by the tissues acropetally, none was found to be translocated through to receiver blocks.

FIG. 14. Tissue radioautographs of sections of Phaseolus multiflorus epicotyl segments after treatment with IAA-H^3 from donor agar blocks (x 0.64). A, diffusion time 1 hr; B, diffusion time 3 hrs; C, diffusion time 1 hour then the donor block was replaced with an agar block containing the same concentration of cold IAA. Left for 2 hrs; D, diffusion time 3 hrs, tissue laid horizontally; E, tissue laid horizontally. Labelled agar blocks replaced after 1 hr with blocks containing cold IAA for 2 hrs; F, epicotyl or internode left attached to seed and decapitated. Labelled agar block placed on cut surface and left 3 hrs; G, same as F but labelled block replaced with block containing cold IAA after 1 hr; Controls, sections treated in same way but fixed instead of developed. In every instance the sections to the left were those nearest to the donor block (1.25 mm from it); those on the right farthest away (3.75 mm from it). The sections from D and E were placed so that their lower sides during treatment were to the left on the slide.

I II

FIG. 15. Tissue radioautographs of sections of Phaseolus multiflorus epicotyl segments after treatment with IAA-H^3 from donor agar blocks (x 7.1). I, detail of figure 14, slide A. II, detail of figure 14, slide E.

Little tryptophan-C^{14} was translocated through the segments either acropetally or basipetally at either 10^{-5}M or 10^{-4}M donor concentrations. Even after 6 hrs with the 10^{-4}M concentration (16,800 cpm/donor block) on Runner bean epicotyl segments, a very small percentage of that applied in the donor blocks reached the receivers. These results are in keeping with results obtained by Schrank and Murrie (1962) for oat coleoptiles.

In Section A of the thesis it was found that tryptophan was the major endogenous indole compound diffusing both acropetally and basipetally out of excised epicotyls. In Section B when labelled tryptophan and IAA were injected into endosperms of corn and cotyledons of Runner beans, both were found to be translocated acropetally. On the other hand, in this section when tissue segments were used, neither compound was translocated acropetally, and IAA was translocated basipetally but tryptophan was not. These conflicting results may be due to the different techniques employed. Moreover, the fact that tryptophan was not translocated at all in tissue segments may be taken to indicate that it is not involved in an active transport mechanism like that of IAA.

One cannot rule out the fact that substances which diffuse out of cut surfaces may not truly represent what is being translocated. In donor blocks containing tryptophan-C^{14} other labelled compounds were extractable after the blocks had been in contact with the tissue for 3 - 6 hrs. This could have been due to tryptophan diffusing into the tissue, being metabolized and the metabolites diffusing back into the blocks. Or it could have been due to soluble enzymes diffusing into the agar blocks.

The dye translocation experiments shed some light onto the diffusion through the segments of compounds which are not actively translocated. That the dye was translocated through the xylem vessels was not surprising for in freshly excised segments, as are used for translocation studies, the xylem vessels are filled with fluid. Such a movement of soluble materials by diffusion in the static cylinders of liquid would be expected to occur and be non-directional. In translocation experiments of long duration and especially using high concentrations of soluble substances, it would not be surprising to find traces of these substances in the receiver blocks which had moved from the donor blocks simply by diffusion. This could possibly have occurred in the experiments with high concentrations of tryptophan-C^{14} in the present study. The passive uptake of materials from donor blocks into the tissue generally for a limited distance from the source was also shown.

The translocation of dye in the xylem of excised coleoptiles with intact tips was much more rapid than occurred in excised segments. However, the excised coleoptiles were not kept in covered Petri dishes though they were kept in a humidified atmosphere. If the humidity was lower than in a covered dish, the increase in transpiration could have caused the increase in rate of translocation, but because of the efficient method of humidifying used, this is unlikely. In view of the passive translocation by fluid-filled xylem, the importance of a high relative humidity in translocation experiments is emphasized. If the humidity is low, evaporation would be greater from the upper block since the lower one has one side in contact with the surface on

which it stands. The effect of reduced relative humidity could cause an effect similar to the effect of transpiration and cause upward movement in the xylem. However, the increased rate of dye translocation in excised coleoptiles with intact tips could have been a consequence of the apices remaining intact, rather than a consequence of increased transpiration.

Although poor tissue sections were obtained, the autoradiographic study indicated that the IAA-H^3 from donor blocks was present in all types of tissue following administration. There appeared to be localization of label in epidermal, cambial and vascular tissue, though because of the inferior quality of the sections obtained, not much significance is attached to this finding. The fact that label was present in the xylem in highest concentrations was probably an artifact for reasons explained in connection with the dye experiments.

SECTION D. METABOLIC STUDIES

This section deals with studies undertaken to compare the ability of the shoot with that of the storage tissue of the seed to convert tryptophan to IAA. Since the rate of this conversion is slow, relatively long incubation times are necessary.

Sliced Runner bean seedling tissue was incubated with 100 ml of solutions of IAA precursors in distilled water in the dark at room temperature for 24 hrs. When the solutions were examined, it was found that there was bubbling and turbidity due to fermentation as a result of microbial contamination.

Incubating a plant extract or an aqueous suspension containing plant material at room temperature under aerobic conditions provides an excellent environment for the growth of many species of aerobic micro-organisms. In view of the fact that many commonly occurring micro-organisms, such as Escherichia coli and Pseudomonas fluorescens, are relatively efficient in the conversion of tryptophan and other precursors to IAA (Stowe, 1955) investigations into microbial contamination and its avoidance were conducted.

To determine the numbers of micro-organisms present in an incubation mixture when taking usual microbiological laboratory precautions, the following experiment was designed.

Runner bean shoots, approximately 12 cm long, were rinsed three times in sterile water. The apical 2 cm of epicotyls with leaves were cut off with flamed scissors into a mortar. The mortar and pestle

had previously been sanitized with a 10% solution of commercial sodium hypochlorite (Perfex) for 10 min at room temperature, and rinsed several times with sterile water. The tissue was ground in sterile buffer (M/100 KH_2PO_4 + 1% sucrose, pH 4.7, Larsen, 1949), transferred to a sterile Erlenmeyer and made up to 100 ml with sterile buffer. At intervals samples were removed, diluted serially in 9 ml sterile water blanks and plated aseptically using Difco Nutrient Agar. The Petri dishes were incubated at room temperature in the dark for 2 days and the colonies counted. Plates with 30 - 500 colonies were used in the calculations since this is considered the most accurate range. The results are shown in figure 16.

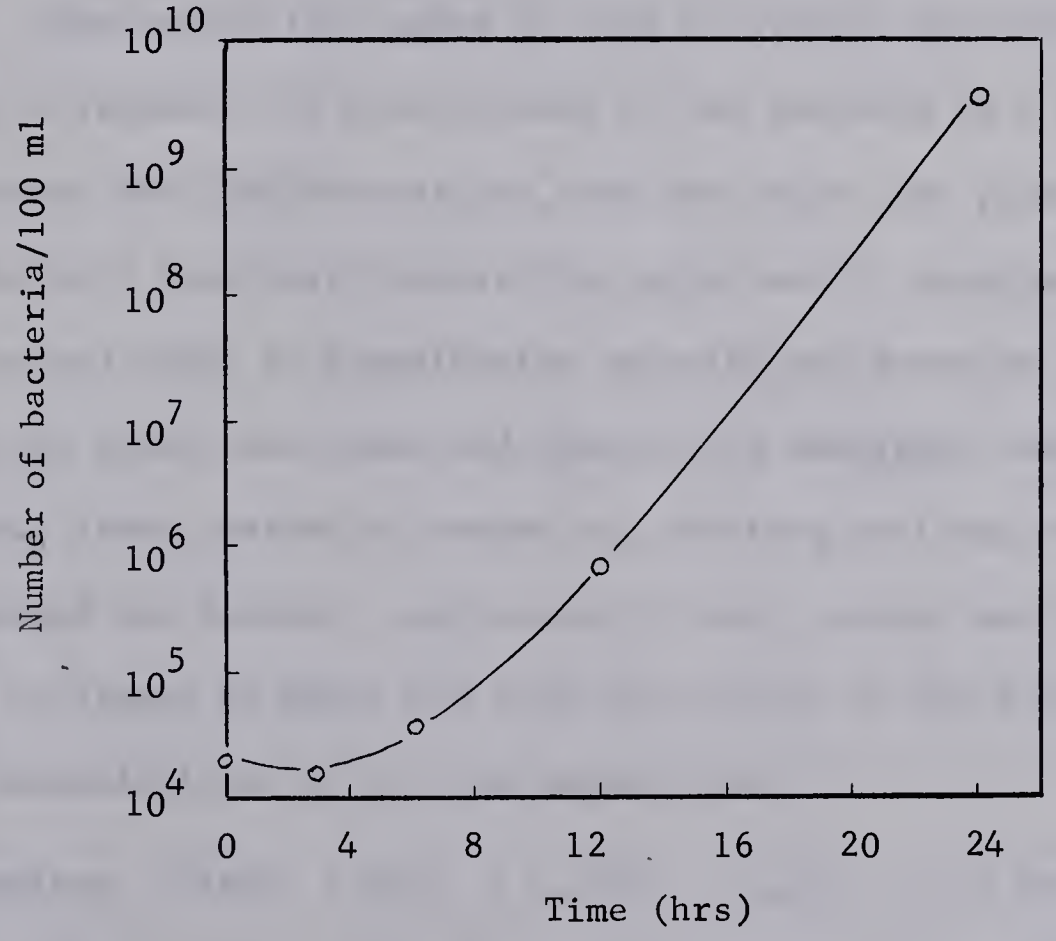
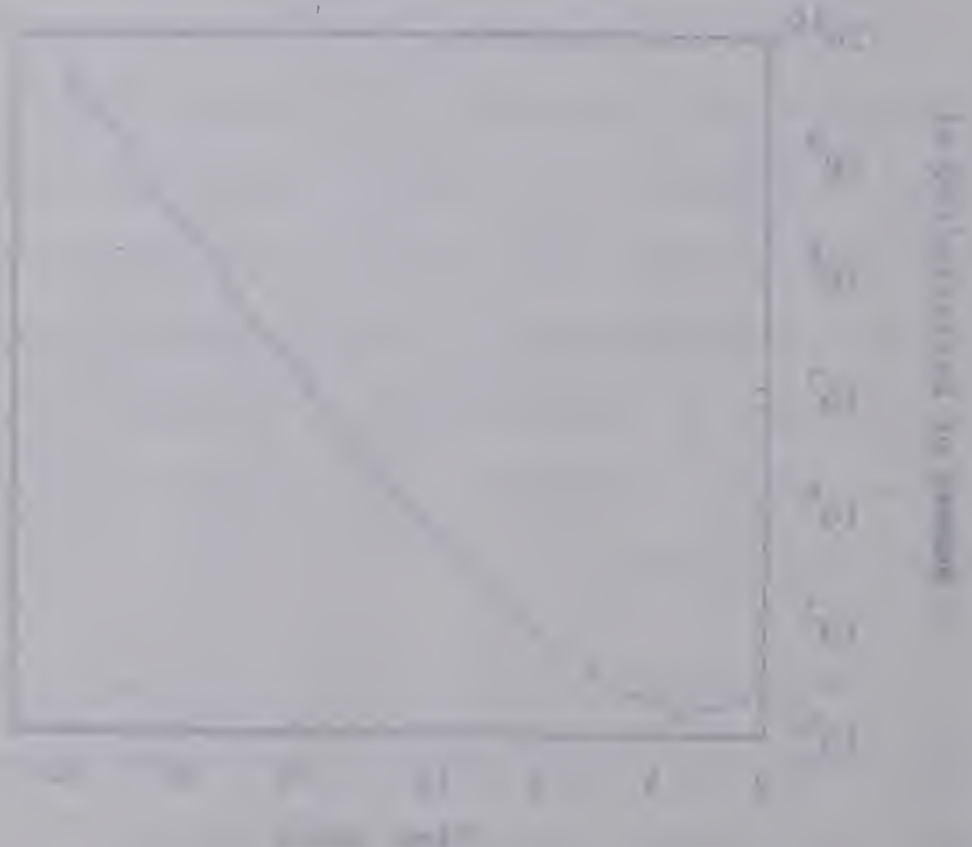


FIG. 16. Growth curve of micro-organisms in plant tissue preparation from prewashed Phaseolus multiflorus shoots.



Colonies on the plates of the shorter incubation times (or lower dilutions) were mainly fungal whereas the others were bacterial. Microscopic examination of bacteria from colonies picked at random from the plates showed that all were Gram negative rods. Further identification was not carried out.

Thus even when great care was taken during preparation, very considerable microbial contamination occurred. Many incubations were carried out for 12 hrs using macerated tissue and in every case the suspensions were plated out for bacterial numbers before being freeze-dried. As all were heavily contaminated, the results on metabolism were not used.

One method that might be used to prevent microbial contamination is to incubate the plant tissue in the presence of a bactericide. This assumes that the bactericide does not affect the plant material. Two relatively non-toxic bactericides were tested, Zephiran (Winthrop Laboratories) which is benzalkonium chloride and Panacide (B.D.H.). Six-day old Dutch brown bean and Runner bean seedlings were harvested above soil level, washed to remove any adhering soil and cut into small pieces and pooled. Approximately equal amounts were added to each of 10 flasks to which had been added 90 ml of the following serial concentrations of the two bactericides:

(a) Zephiran 1:100; 1:300; 1:1,000; 1:3,000; 1:10,000

(b) Panacide 1:300; 1:1,000; 1:3,000; 1:10,000; 1:30,000

These were stoppered with cotton wool and incubated at room temperature (25^{o} C) in the dark for 24 hrs. One ml samples were

plated out aseptically using Difco Nutrient Agar. It was found that Zephiran 1:300 or higher and Panacide 1:1,000 or higher inhibited microbial growth.

To determine if Runner bean seedling tissue could metabolize tryptophan in the presence of Zephiran or Panacide and also to see if micro-organisms present on the plant surface will metabolize tryptophan, the following experiment was set up using tissue slices excluding the hypocotyl and root:

(1) tissue with 100 ml 1:1,000 Panacide

(2) tissue with 100 ml 1:300 Zephiran

(3) tissue with 100 ml distilled water

(4) tissue autoclaved in 100 ml distilled water, cooled and inoculated with 10 ml of a washing of another plant in sterile water.

Thirty µl (0.6 µc) of tryptophan-C^{14} were added to each flask. These were incubated in the dark for 48 hrs after which time the contents of the flasks were freeze-dried, extracted with methanol and chromatographed.

With Panacide, no metabolic products were detected. With Zephiran it was difficult to discern whether metabolism occurred as there was streaking on the chromatograms. Without disinfectant several compounds were detectable and there was little tryptophan left. With the autoclaved plant tissue to which a washing of another plant was added, there was also some metabolism of the tryptophan. Thus it was demonstrated that micro-organisms washed from the surface of plant tissue metabolized tryptophan.

If seedling tissue could be surface-sterilized to destroy all contaminating micro-organisms including the resistant spores of spore-formers without the sterilant penetrating or damaging the plant material, the problem of microbial contamination would be readily solved. However, several treatments which were tried to surface sterilize the shoot tissue including Zephiran 1:300 for 5 min, 70% ethanol for 1-5 min and sodium hypochlorite 10% for 5 min did not accomplish these objectives.

Taking all factors into consideration perhaps the safest approach when using plant tissue in metabolic studies is to circumvent serious microbial contamination by using short incubation periods. Direct injection of metabolites into the tissues or allowing excised plants to take up the metabolites for short periods may also be used.

Tryptophan metabolism in shoots and cotyledons was investigated in this way. Intact Runner bean seedlings were injected with 3 - 5 µl quantities of tryptophan-C^{14} (0.1 µc/5 µl) per cotyledon. After 20 - 24 hrs the growing tips, epicotyls and cotyledons were extracted separately with methanol and the extracts chromatographed in two solvent systems. Also excised epicotyls were placed into vials (3/vial) containing 40 µl tryptophan-C^{14} (0.8 µc) in 1 ml of distilled water. The following day distilled water was added to replace the solution which had been taken up by the plants. After 20 - 24 hrs the basal parts of the epicotyls which were dipping into the solutions were removed and the growing tips and epicotyls extracted separately with methanol and the extracts treated as above. The R_f values of labelled regions on the chromatograms detected using both scanning and radioautograph procedures are given in table XVIII.

Only one major metabolite of tryptophan was detected. It had a mean R_f of 0.12 in isopropanol:ammonium hydroxide (28%):water, and 0.77 in isopropanol:acetic acid:water. For both treatments the concentrations of this metabolite isolated from the growing tips were approximately the same as or higher than that of tryptophan-C^{14}, whereas in the other plant parts tryptophan-C^{14} was in a higher concentration. This experiment was repeated with similar results.

Table XVIII. Major radioactive regions on chromatograms of methanol extracts of Phaseolus multiflorus seedling tissue which had been treated with tryptophan-C^{14} (a) by allowing the epicotyls to take up the solution and (b) by injection into cotyledons

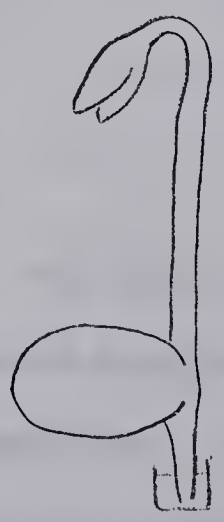
Method of applying tryptophan-C^{14}		Tissue extracted	R_f of radioactive regions on chromatograms developed in isopropanol:NH_3:water, 8:1:1		R_f of radioactive regions on chromatograms developed in isopropanol:acetic acid:water, 4:1:1	
(a)	solution	shoot tips	0.09*	0.26	0.41	0.81
		epicotyls	0.12	0.32*	0.39*	0.75
(b)	injected	shoot tips	0.11*	0.29	0.35*	0.70
		epicotyls	0.14	0.32*	0.47	-
		cotyledons	0.13	0.33*	0.38*	0.72
	Mean R_f of metabolite		0.12	0.30	0.40	0.77
	Tryptophan-C^{14}			0.28	0.35	

* greater amount as evidenced from Actigraph charts and radioautographs.

This procedure was further investigated by administering high concentrations of cold tryptophan in addition to labelled tryptophan to enhance metabolism (Wightman, 1964).

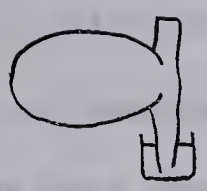
Seven-day old dark-grown Runner bean seedlings were fed in this way. Two concentrations of cold tryptophan were investigated, 10^{-3}M and 10^{-2}M. The solutions were prepared in phosphate buffer of pH 7.0 (50 ml 0.1 M KH_2PO_4 + 29 ml 0.1 M NaOH) + 1% sucrose. A small amount of ammonium hydroxide was used to dissolve the tryptophan and this was neutralized with dilute hydrochloric acid before the buffer was added.

The following treatments were employed:



I. Seedlings were cut under water just below the hypocotyls. The cut ends were dipped into 0.6 ml of the tryptophan + buffer solution to which 50 µl of tryptophan-C^{14} (1 µc) were added. Three plants were used per treatment. After treatment the epicotyls plus leaves and cotyledons were extracted separately.

II. Epicotyls were excised under water just above the cotyledons. Three epicotyls were placed into each vial containing 1 ml of the tryptophan + buffer solution and 75 µl of tryptophan-C^{14} (1.5 µc). After treatment the lower 2 cm of the epicotyls were removed, and discarded.



III. Epicotyls and roots were excised under water so that the cotyledons were attached to small pieces of epicotyls and hypocotyls. The hypocotyls were dipped into vials containing 0.6 ml of tryptophan + buffer solution and 40 µl of tryptophan-C^{14} (0.8 µc). There were 2 plants per treatment.

The plants were kept in the dark at room temperature and a fan directed towards the plants to increase transpiration and uptake of the solutions. When most of the solution had been taken up by the plants, after approximately 4 - 8 hrs, more cold tryptophan + buffer was administered. Subsequently distilled water was added when necessary. After 24 hrs the plant parts were ground and extracted with methanol overnight at 3^{o} C. After filtering, the extracts were evaporated to dryness at 45^{o} C under reduced pressure. The subsequent method of partitioning approximately follows that of Wightman (1964).

The samples were dissolved in warm water and filtered through diatomaceous earth (Hyflo Supercel). The solutions were adjusted to approximately pH 7.0 with 1% sodium bicarbonate solution and extracted 4 times with fresh Mallinkrodt diethyl ether. The ether fractions designated NEUTRAL ETHER FRACTIONS were dried over anhydrous sodium sulphate. The aqueous solutions were then adjusted to pH 3.0 - 3.5 with dilute HCl and re-extracted 4 times with ether to yield the ACID ETHER FRACTIONS. Then the aqueous solutions were extracted 4 times with n-butanol to yield BUTANOL FRACTIONS. The remaining aqueous extracts were evaporated to dryness under reduced pressure and re-dissolved in methanol to give the METHANOL FRACTIONS.

All extracts were evaporated down to small volumes, chromatographed and radioautograms made of the chromatograms. Some of the chromatograms were then sprayed with DMAC. The results for the 10^{-2}M and 10^{-3}M tryptophan treatments were similar. The R_f values of labelled compounds on chromatograms of the 10^{-2}M treatments are given in figures 17-20.

Upon careful examination of the figures it may be seen that many metabolites were present. The neutral ether fraction yielded traces of 2 compounds at mean R_f values of 0.75 and 0.88 (mean of 8 extracts). In the acid fractions an intense band at a mean R_f of 0.10 was obtained in all 8 extracts. This region reacted blue-purple with DMAC. Other compounds were detected in only trace amounts. In some cases a compound at an R_f region corresponding to IAA was detected, but without further tests its identity could not be established.

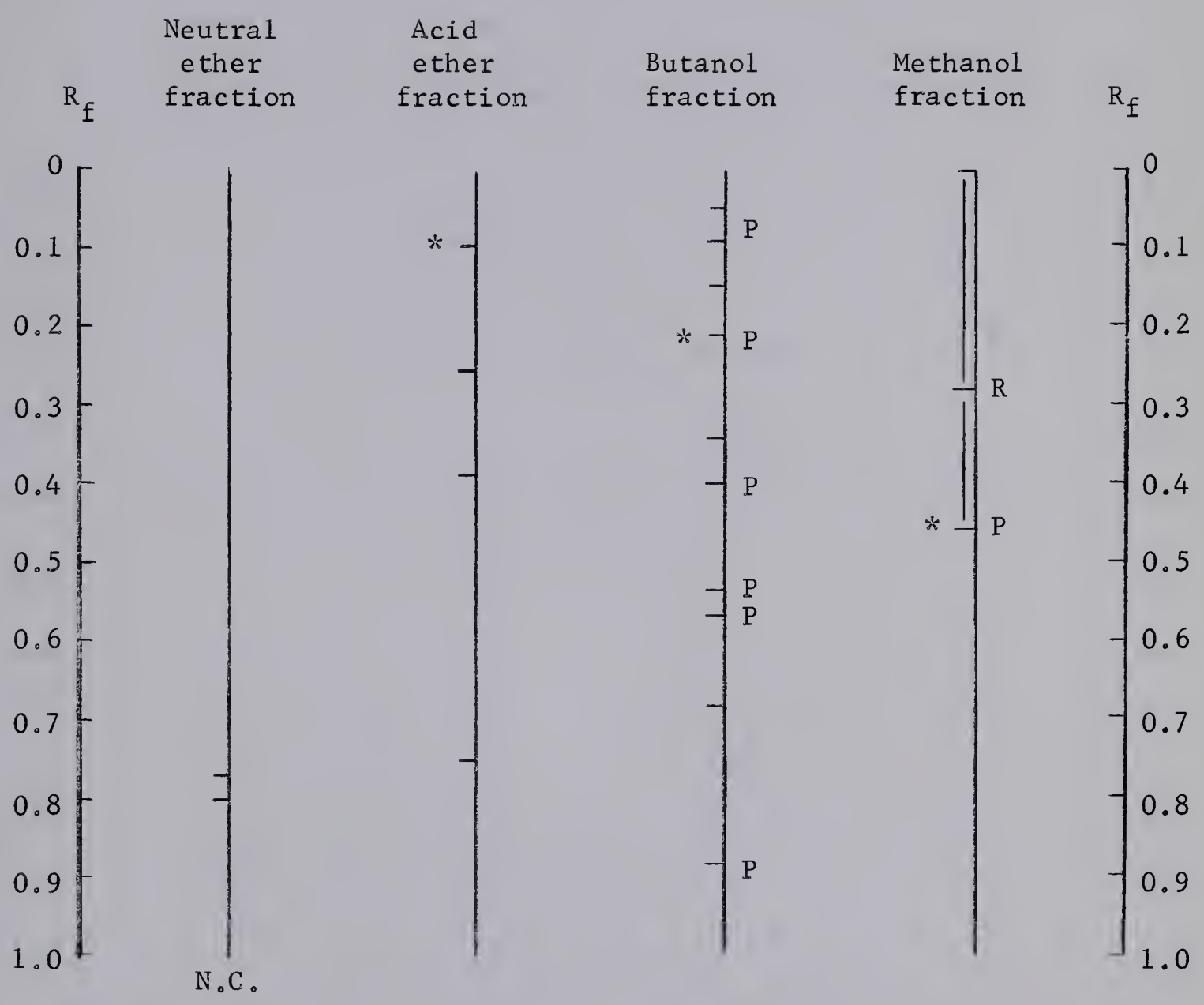


FIG. 17. Mean R_f values of labelled compounds and DMAC reactions on chromatograms of extracts of Phaseolus multiflorus epicotyls and leaves which had been treated with tryptophan-C^{14} in combination with 10^{-2}M tryptophan. Treatment I, solution taken up through hypocotyl of seedling; shoot and cotyledons left intact. (Marks to left of vertical lines indicate labelled compounds; * major compound. Colour reaction with DMAC, P = purple, R = red or pink, B = blue, N.C. = no colour.)

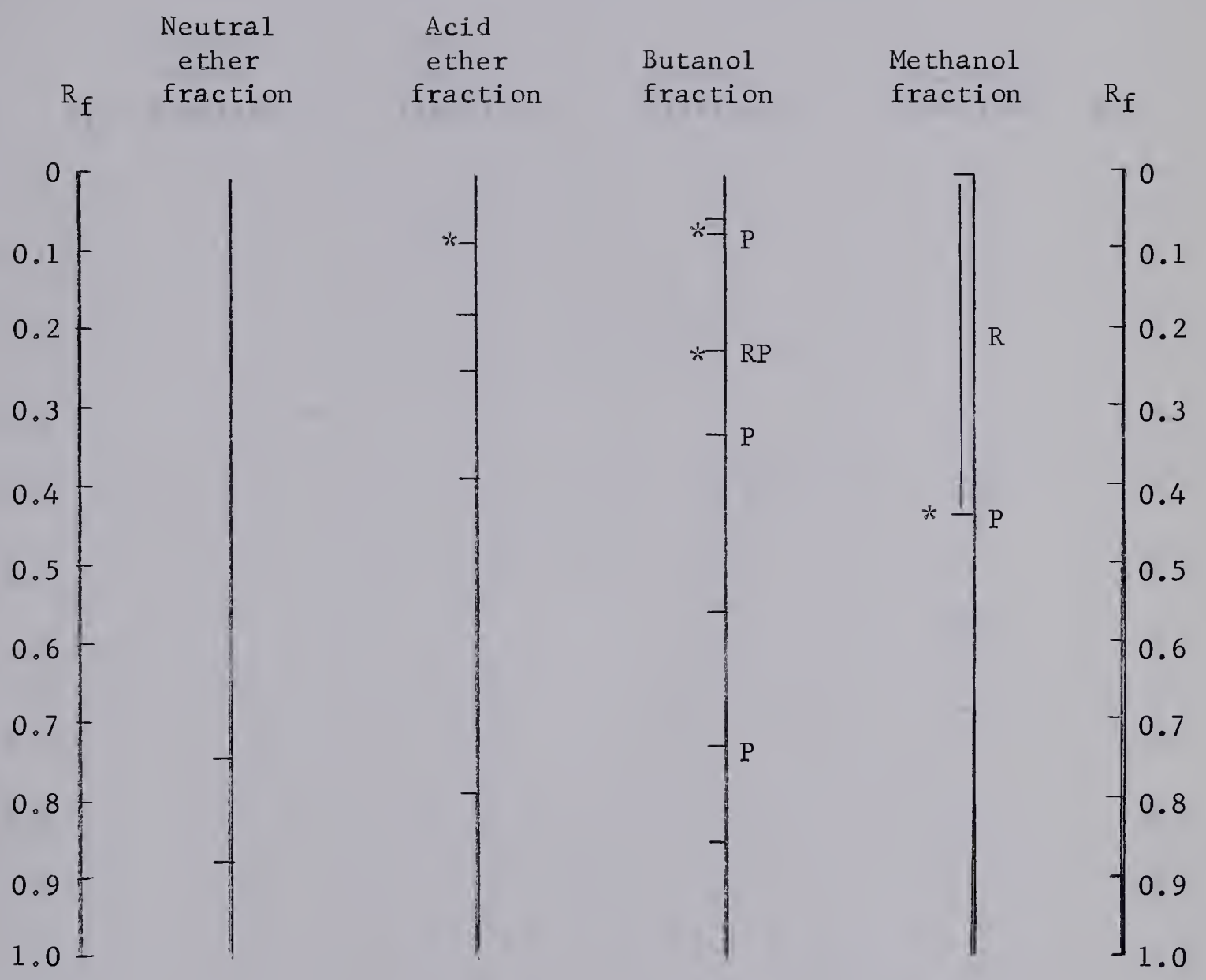
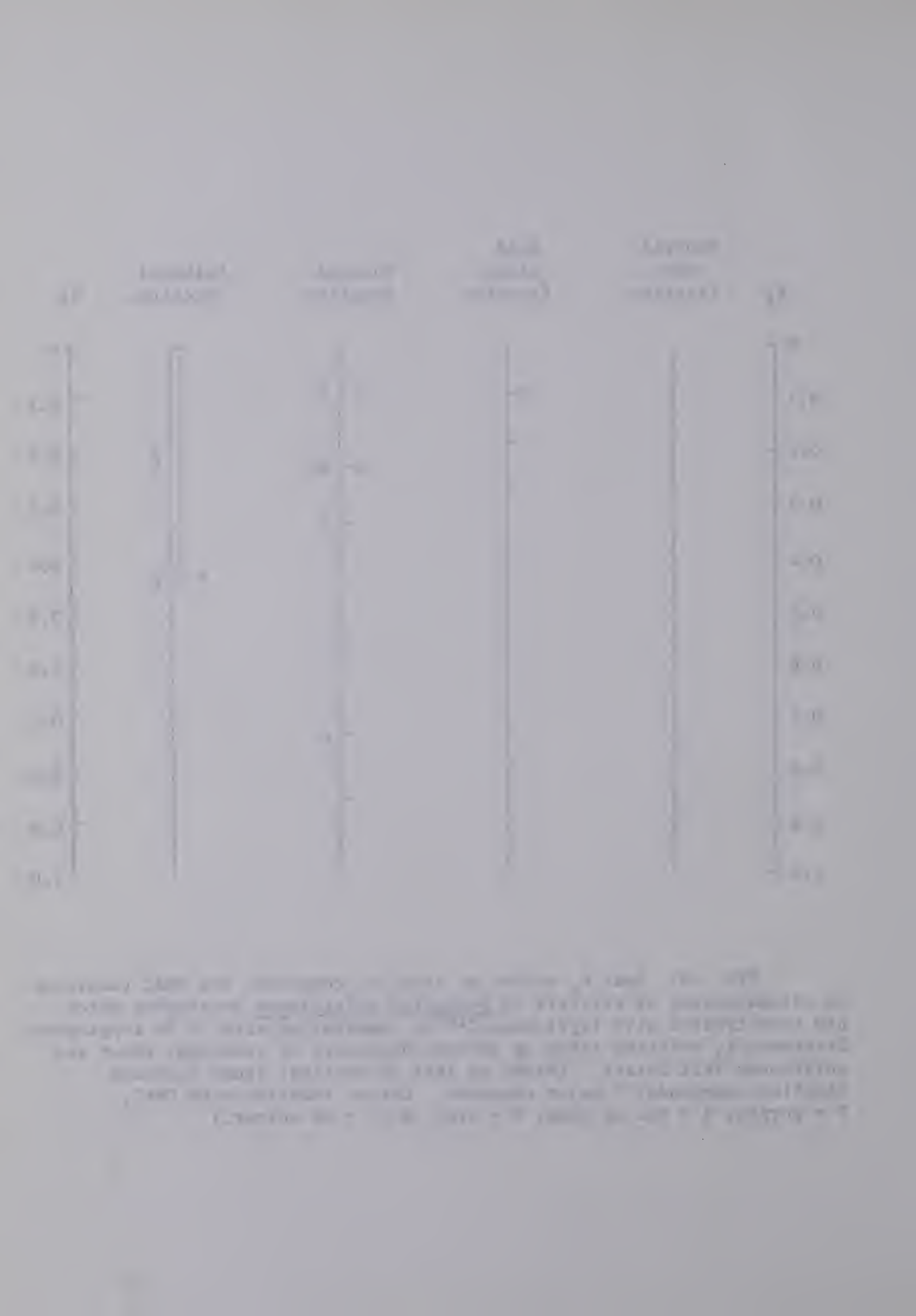


FIG. 18. Mean R_f values of labelled compounds and DMAC reactions on chromatograms of extracts of Phaseolus multiflorus cotyledons which had been treated with tryptophan-C^{14} in combination with 10^{-2}M tryptophan. Treatment I, solution taken up through hypocotyl of seedling; shoot and cotyledons left intact. (Marks to left of vertical lines indicate labelled compounds; * major compound. Colour reaction with DMAC, P = purple, R = red or pink, B = blue, N.C. = no colour.)



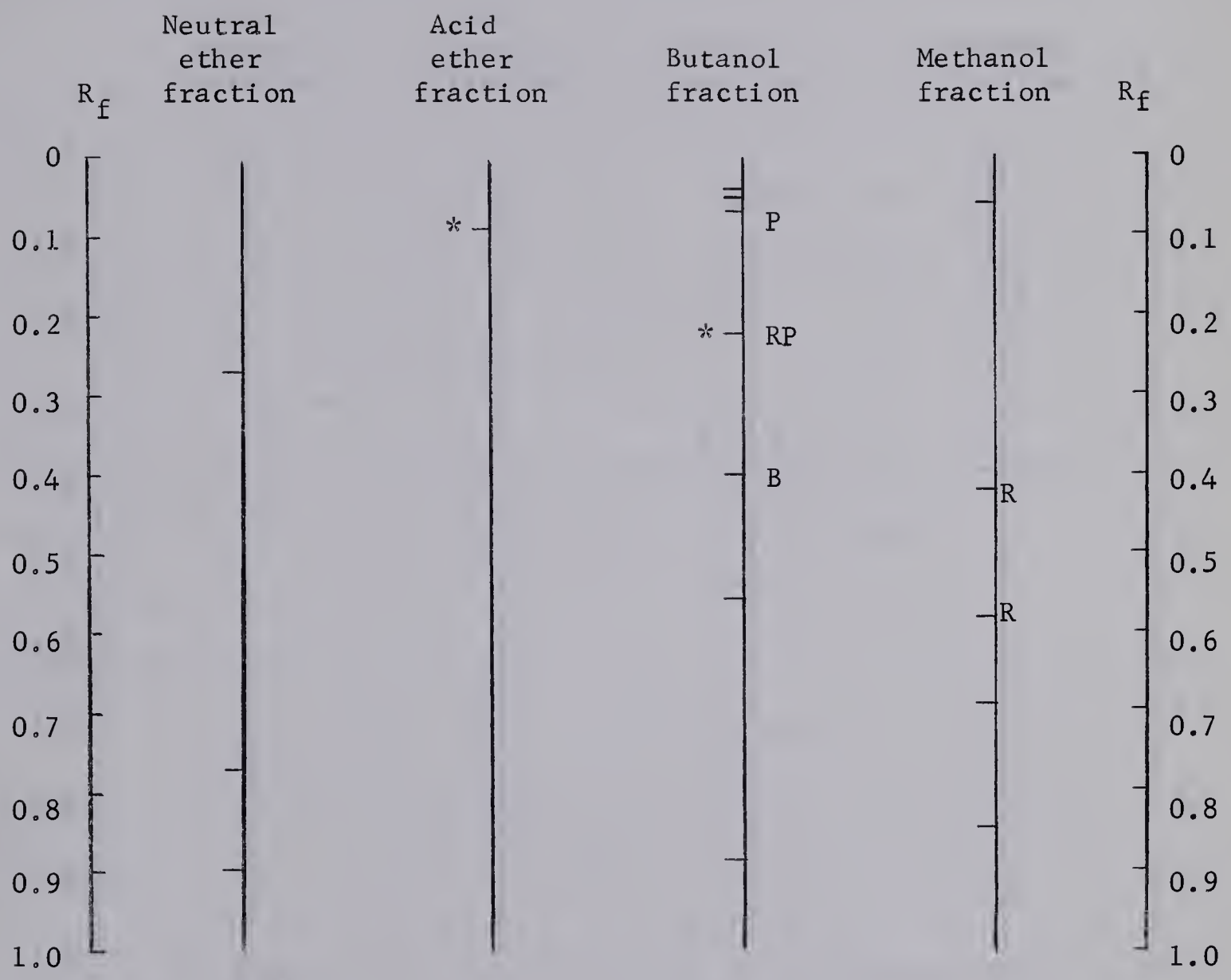
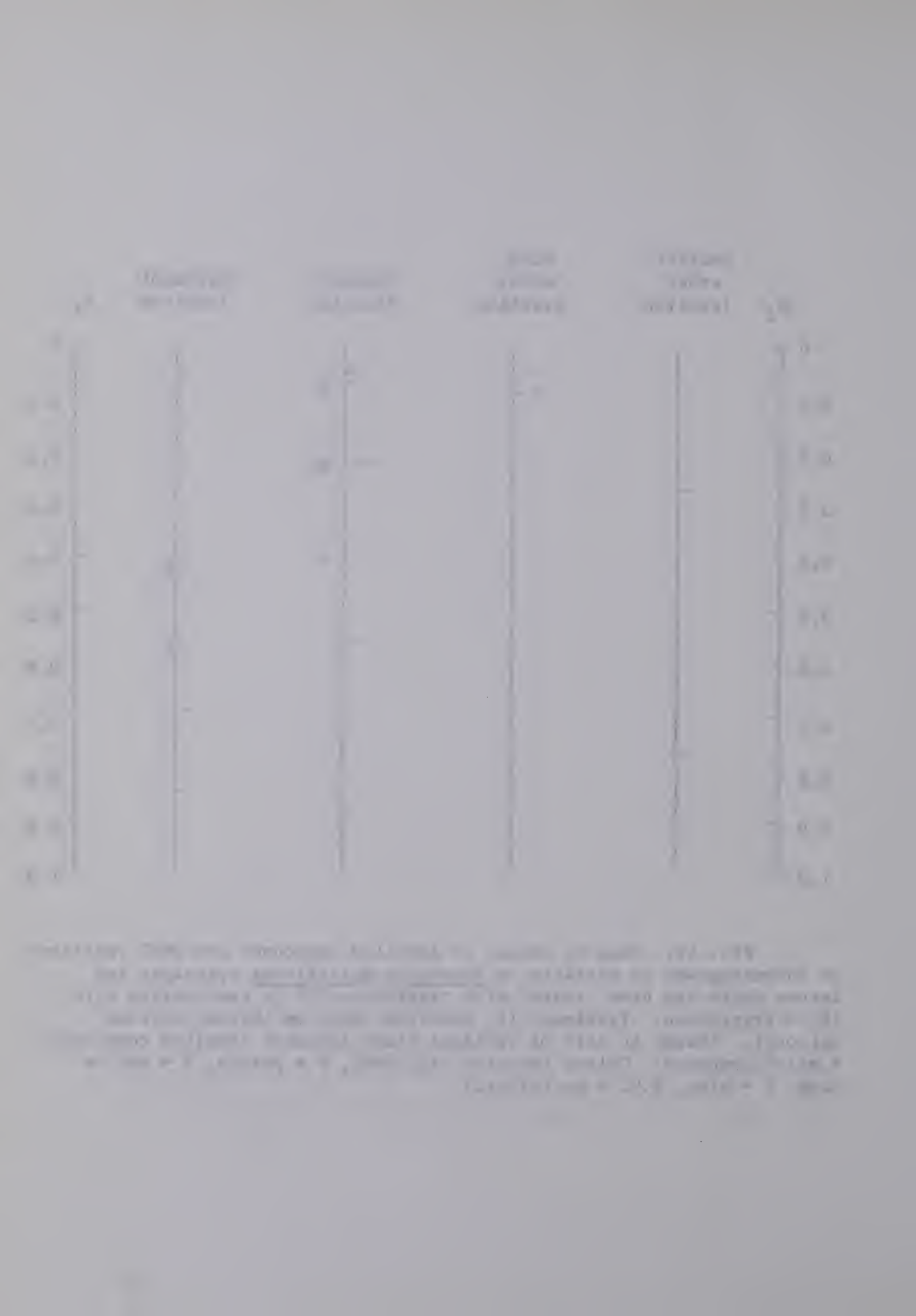


FIG. 19. Mean R_f values of labelled compounds and DMAC reactions on chromatograms of extracts of Phaseolus multiflorus epicotyls and leaves which had been treated with tryptophan-C^{14} in combination with 10^{-2}M tryptophan. Treatment II, solution taken up through excised epicotyl. (Marks to left of vertical lines indicate labelled compounds; * major compound. Colour reaction with DMAC, P = purple, R = red or pink; B = blue, N.C. = no colour.)



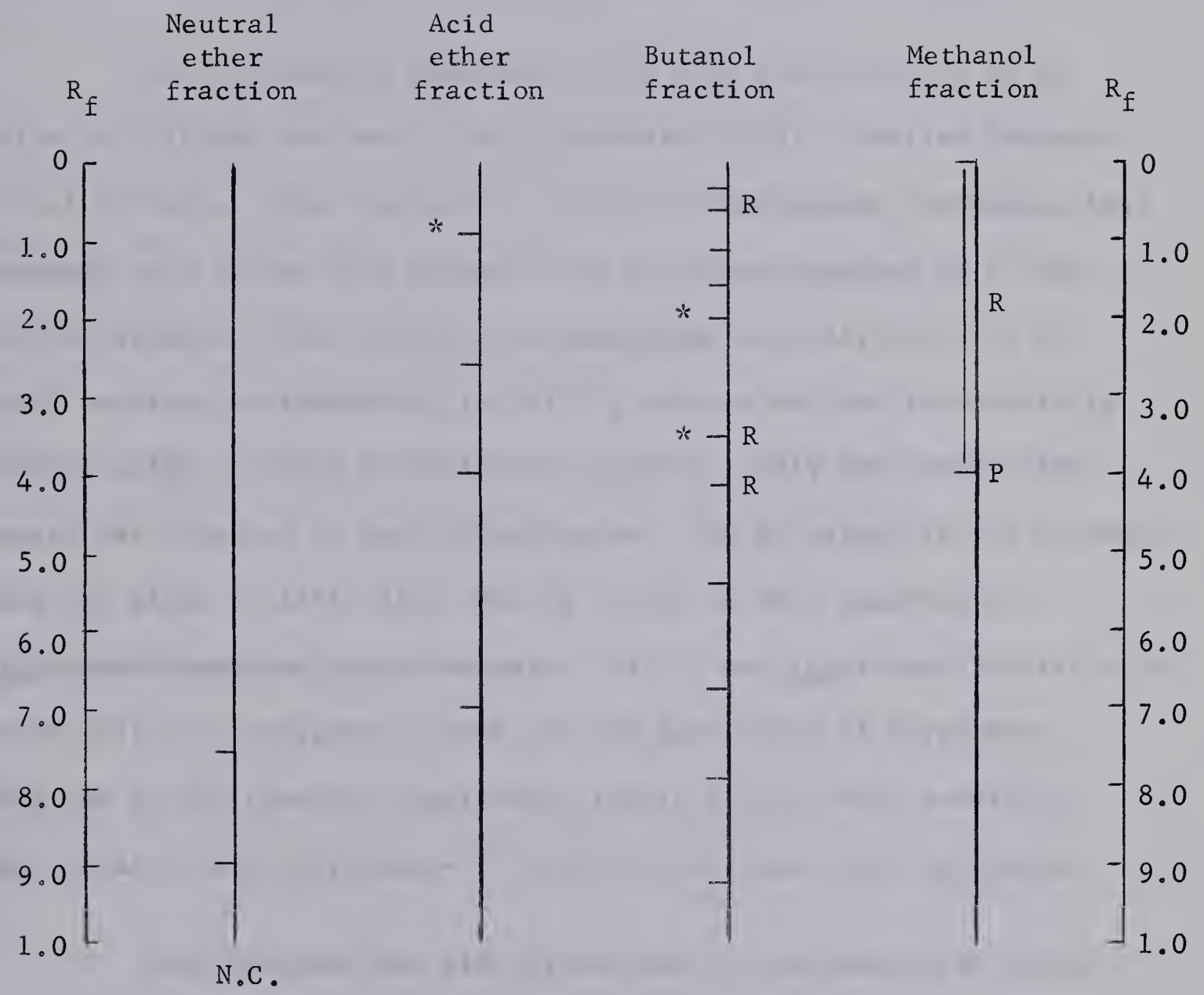
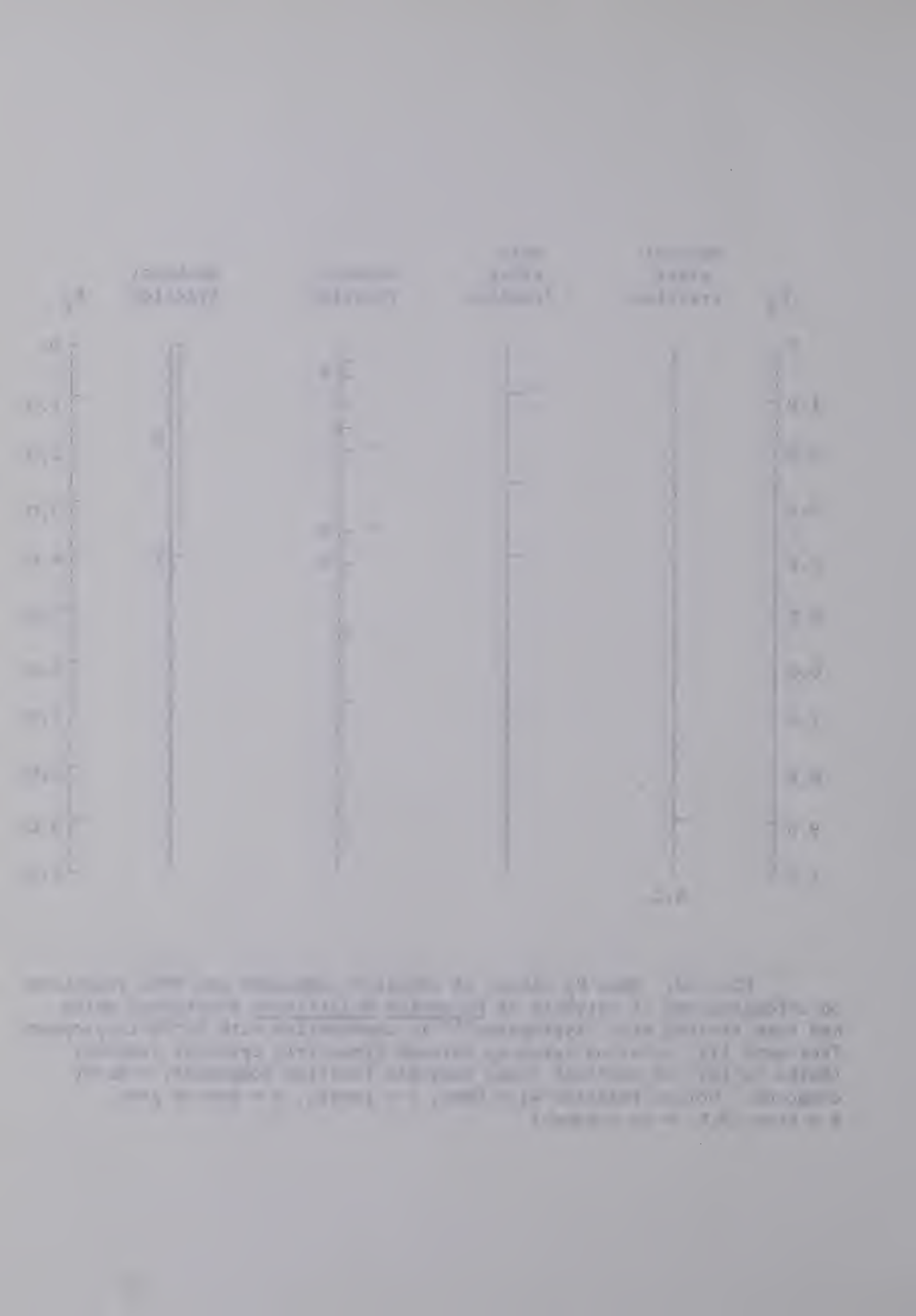


FIG. 20. Mean R_f values of labelled compounds and DMAC reactions on chromatograms of extracts of Phaseolus multiflorus cotyledons which had been treated with tryptophan-C^{14} in combination with 10^{-2}M tryptophan. Treatment III, solution taken up through hypocotyl, epicotyl removed. (Marks to left of vertical lines indicate labelled compounds; * major compound. Colour reaction with DMAC, P = purple, R = red or pink, B = blue, N.C. = no colour.)



Very many labelled compounds were present in the butanol fractions in all cases, the major one being at a mean R_f of 0.23. Much activity remained in the methanol fractions but this generally streaked on chromatography. The major band chromatographed at a mean R_f of 0.42.

The radioactive compound in the acid fraction with an R_f value of 0.10 was the most clearly separated highly labelled compound in all extracts. The regions on 7 of the chromatograms containing this compound were eluted with methanol and re-chromatographed in 4 other solvent systems. The developed chromatograms were divided into 20 equal sections corresponding to half R_f regions and the radioactivity counted using a liquid scintillation counter. Only one radioactive region was detected on each chromatogram. The R_f values in the solvents used are given in table XIX. The R_f values of this compound in isopropanol:ammonium hydroxide:water (8:1:1) and isopropanol:acetic acid: water (4:1:1) correspond to those of the metabolite of tryptophan obtained in the previous experiments (table XVIII) where seedlings were treated with tryptophan-C^{14} without additional cold tryptophan.

This compound was also hydrolyzed by refluxing with 6N HCl for 1 hr. After neutralizing, the extract was evaporated to dryness, re-dissolved in methanol and re-chromatographed in the original solvent. Although a labelled compound was present with an R_f value of 0.08 near that of the original compound (0.10), a second labelled region was also detected (0.30 - 0.50).

Table XIX. Mean R_f values in several solvents of the major labelled compound present in acid ether extracts of Phaseolus multiflorus tissue, after allowing the tissue to take up tryptophan-C^{14} in combination with 10^{-2}M tryptophan

Chromatography solvent*	Mean R_f value
isopropanol:ammonium hydroxide (28%):water (8:1:1)	0.10
isopropanol:acetic acid:water (4:1:1)	0.67
butanol:acetic acid:water (12:3:5)	0.81
butanol:ethanol:water (4:4:1)	0-0.05
8% aqueous sodium chloride (ascending)	0.87

* developed by descending technique except where specified.

Summary and Discussion

The presence of large numbers of micro-organisms in tissue preparations was found to be a problem in incubations of relatively long duration. Metabolism of supplied tryptophan-C^{14} was found to occur when the enzymes of the plant tissue were destroyed and the preparation was inoculated with micro-organisms washed from the surface of plant material. It had previously been shown by Stowe (1955) that a number of commonly occurring bacteria were able to convert tryptophan to IAA. In view of the foregoing, if tissue preparations become contaminated, one would not be able to attribute any metabolism which occurred solely to the enzymes of the plant. Kent and Gortner (1960) found that indoleacetaldehyde was converted to IAA by plant tissue preparations in which enzymes had been destroyed by boiling, and they found heavy

contamination of Gram negative rods. The isolated bacteria were also found to cause this conversion. Kent and Gortner stated that erroneous information may derive from enzyme studies where growth of micro-organisms is not effectively controlled. Studies on metabolism of plant tissue preparations where long incubation times were used, have however been reported by several investigators without their having taken contamination by micro-organisms into account.

Several methods of reducing contamination without affecting enzymatic activity were tried but none was successful. In view of the characteristic growth curve of a culture of micro-organisms (Thimann, 1963b and fig 16) incubations of the order of a few hours are not so prone to this problem, for when a culture of micro-organisms is transferred to a new environment very little growth and multiplication occurs for a few hours (lag phase). After this time, however, the organisms reproduce at a logarithmic rate, the generation time for many micro-organisms being less than 30 mins.

Bacon et al. (1965) were able to obtain aseptic conditions in their studies with storage tissue discs, but this is easier to accomplish with such material than with the material used in this study. In studies with storage tissue discs, Leaver and Edelman (1965) found difficulty in achieving the aseptic conditions obtained by Bacon et al. and resorted to the use of antibiotics to prevent microbial growth in their preparations. They found that chloramphenicol controlled microbial growth but did not affect the enzymes they were studying. In the present study no metabolism of tryptophan-C^{14} occurred when tissue

was incubated in the presence of 2 bactericides at concentrations which would inhibit microbial growth.

The technique finally adopted in the present study was to administer the labelled precursor to plants for a relatively short time and subsequently to administer unlabelled precursor or water. Without the administration of unlabelled tryptophan it was found that only one metabolite of the tryptophan-C^{14} was detected. However, when unlabelled tryptophan was also fed, many metabolites were found. Further investigation would be necessary to identify the metabolites and to obtain quantitative data to compare the metabolic abilities of the shoot and cotyledons.

One acidic compound which was consistently detected in all experiments (including the earlier ones where tryptophan-C^{14} alone was fed) was the one for which R_f values in several solvents are given in table XIX. In view of its low R_f value in isopropanol: ammonium hydroxide:water, it was considered as a conjugate. Unfortunately no synthetic conjugates were chromatographed with this compound. It possessed similar R_f values to indoleacetylaspartic acid in only 3 of the 5 solvent systems used. Since DL-tryptophan-C^{14} was administered the possibility of its being malonyl-D-tryptophan arises. Zenk (1964), who investigated this compound, used thin layer chromatography and so no direct comparison between our R_f values may be made. Hydrolysis of the compound was found to yield 2 labelled compounds.

GENERAL DISCUSSION AND CONCLUSIONS

Although discussions have already been included, it seems desirable to relate the major findings of the separate sections. This is particularly important in order to explain the seemingly different results obtained in Sections A, B and C relating to translocation.

Tryptophan was found to diffuse out of excised epicotyls of Runner bean seedlings both acropetally and basipetally in Section A. In Section B the acropetal transport of injected tryptophan-C^{14} was also shown. However, in Section C no translocation in either direction occurred using tissue segments. Injected IAA-C^{14} was found to be translocated acropetally in Runner bean and corn shoots in Section B whereas in Section C no acropetal translocation was found. The discrepant results must be consequences of the various techniques employed. Methods in Section C test only active translocation such as occurs with IAA. From the results obtained it was inferred that tryptophan is not involved in such a transport mechanism. In intact seedlings factors, such as root pressure, diffusion pressure deficit, etc., might be influencing the acropetal translocation of injected IAA-C^{14} and tryptophan-C^{14} observed in Section B. In excised epicotyls, interference with the natural balance of pressures occurs and this must be taken into consideration when comparing the compounds present in exudates with those being translocated naturally. Although tryptophan-C^{14} was found not to be actively translocated in Section C experiments, it is still

probable that it could be the common precursor of IAA which is translocated from storage tissue of the seed to the actively growing, IAA producing, regions of the shoot, as evidenced from Section A.

It is possible that several alternative precursors and pathways may be used for the production of IAA by the tips, involving both tryptophan and other IAA precursors or conjugates (Gordon, 1961).

On the basis of several tests, including co-chromatography in 4 solvents, its elution time on an ion exchange column and column co-chromatography with labelled and cold tryptophan, the major indole compound extracted from Phaseolus multiflorus cotyledons and shoots was considered to be tryptophan. Its concentration in cotyledons as determined by the amino acid analyzer was found to be ca. 70 µg/g fresh weight.

From injection of IAA-C^{14} into Runner bean cotyledons and corn endosperms, any transport of IAA conjugates or precursors to the shoot was obscured by the fact that IAA-C^{14} itself was translocated. In view of the fact that others have been unable to collect even traces of endogenous IAA diffusing acropetally from excised seedling shoots, the acropetal translocation of injected IAA-C^{14} must tentatively be considered as an unnatural phenomenon. However, it does represent another instance of acropetal transport of IAA. Although an extensive vascular system was found in cotyledons of Phaseolus multiflorus and Phaseolus vulgaris, it was concluded that the acropetal transport did not occur as a result of direct injection into the xylem.

The fact that the dye, Light Green, and radioactivity were found to be translocated in segments of tissue through the xylem vessels, indicates that small amounts of applied substance, present in receiver blocks, may not be significant if high concentrations in donor blocks and long diffusion times are used. Also uptake in the tissue of soluble materials from donor blocks is not significant in itself, since as had been shown and pointed out by Oserkowsky (1942) and Thimann (1964), this may be merely a diffusion into cell walls and "free" space. Donor blocks initially containing tryptophan were found to contain traces of other compounds after being in contact with tissue segments. The other compounds, present in larger amounts after 6 hrs than after 3 hrs may be a consequence of metabolism by the tissue and diffusion back into the blocks. In view of the short time of the treatment, metabolism due to microbial growth is unlikely.

The techniques used in preparation of the tissue autoradiographs proved to be difficult and tedious, but they could probably be refined to produce better results.

Although the autoradiograms of tissue after treatment with IAA-H^3 were poor, they are, to the author's knowledge, the only direct evidence of this type so far reported. They directly indicate that IAA is translocated in all types of living tissue as has been stated by Went and Thimann (1937) and Leopold (1955, 1961) from other evidence.

Although one of the initial objectives was to compare the abilities of the cotyledons and endosperms with the shoot tips for

the conversion of tryptophan to IAA, this was not realized because most of the results from the metabolic studies were regarded as spurious due to the high incidence of microbial contamination.

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